

**INTERACTIONS BETWEEN *PHYTOPHTHORA CINNAMOMI*,
PYTHIUM ULTIMUM, *PSEUDOMONAS CEPACIA* AND SEEDLINGS**

By

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I certify that this thesis, except where assistance is acknowledged,
is my own work.

A handwritten signature in blue ink, appearing to read 'Li Dongping', written in a cursive style.

Dong-ping Li, December 1990.

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ABSTRACT

This thesis reports a study of the interactions between seedlings, the plant pathogenic fungi *Phytophthora cinnamomi* and *Pythium ultimum*, and a potential disease biological control bacterium *Pseudomonas cepacia*, and the influence of soil temperatures and soil matric potentials on the interactions.

Experiments were conducted in a glasshouse with no temperature control and in a constant temperature room. In the glasshouse, complex interactions existed between air temperature, soil matric potential and soil temperature so that under a particular range of air temperatures, the temperatures of soils at various matric potentials were different. These interactions were important in the occurrence of disease caused by *Ph. cinnamomi*.

Ph. cinnamomi produced sporangia and zoospores only in saturated soil, even when soil temperatures were optimal. Its mycelial activity was not directly influenced by soil matric potentials, but was greatly affected by soil temperature. *Ph. cinnamomi* caused severe damping-off and reduced plant weight at all matric potentials tested at 23.5 to 26°C, but did so only in saturated soil when soil temperatures were higher.

Within the range of soil temperatures and soil matric potentials used, mycelial growth of *Py. ultimum* and the severity of turnip damping-off caused by the fungus was not affected.

Turnip seedling survival and plant weight were significantly reduced in saturated soil, indicating a plant stress. Such stress possibly contributed to a higher incidence of disease caused by *Ph. cinnamomi* in saturated soil

The disease control effects of *Ps. cepacia* 526 depended on the specific mutual interactions between the bacterium and the target fungi. *Ph. cinnamomi* inhibited *Ps. cepacia* 526 in both agar and liquid media.

~~Circumstantial~~ ^{Indirect} evidences strongly suggested that the inhibition was related to the production of short-lived antibiotics by the fungus in *in vitro* tests, but attempts to isolate and characterize the antibiotics were not successful. Nutrient contents of the media were critical for the occurrence of this inhibition and thiamine was the essential component. Inhibition by *Ph. cinnamomi* probably stopped the bacterium from moving and multiplying along the fungal hyphae in culture, and minimized the effects of the bacterium on the fungal mycelial growth and sporulation in both culture and soil, and on the disease control.

Py. ultimum did not inhibit the growth of *Ps. cepacia* 526, but was itself inhibited by the bacterium in both agar and liquid media. Quick movement and multiplication of the bacterium along the fungal hyphae occurred in culture, with consequent increase of the fungal hyphal lysis and effective control of damping-off caused by the fungus in soil.

Ps. cepacia 526 was able to establish itself in the rhizosphere soil and on *P. radiata* roots. It competed effectively with the soil bacteria to form the major part of the total microbial population on turnip roots and effectively promoted turnip seedling growth.

The interactions between the fungi and the bacterium and the plant growth-promoting effects of *Ps. cepacia* 526 were not affected by the soil temperatures and soil matric potentials tested, indicating good adaptability of the bacterium within these ranges.

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CHAPTER 1. GENERAL INTRODUCTION

1.1. The importance of controlling diseases caused by *Phytophthora cinnamomi* and *Pythium ultimum* by biological means.

Since it was first isolated by Rands in 1922 from cinnamon trees on which it caused a stripe canker, *Phytophthora cinnamomi* Rands (*Ph. cinnamomi*) has been recognized from over 60 countries as the cause of a variety of diseases of over 900 plant species (Zentmyer 1980). The fungus has become one of the most notorious and widespread pathogens of woody plants in mild temperate, subtropical, and tropical areas. Examples of severe diseases include the root rot disease from which the avocado industry in most subtropical areas ~~have~~ ^{has} suffered great losses, the dieback of avocado in South Africa, pineapple wilt in Queensland, littleleaf disease of *Pinus echinata* in the southern United States, root and collar rot of *Pinus resinosa* in the eastern United States, root and crown rot of *Rhododendron ponticum*, *R. californicum* and *R. carolinianum* (rhododendrons), and the root rot of *Erica gracilis* (heather) in Germany (Zentmyer 1980). *Ph. cinnamomi* also created serious problems of plant decline and death in nurseries in California. Plants commonly affected included species of *Erica*, *Camellia*, *Myrtus*, *Pinus*, *Calocedrus* and *Chamaecyparis* (Zentmyer 1980).

In Australia, *Ph. cinnamomi* has destructively invaded open forest, woodland, and heathland (Weste & Marks 1987). The pathogen was first isolated ^(W.A.) from the jarrah forest (*Eucalyptus marginata*) in Western Australia, then from dying native plants in the eastern forests in New South Wales, and from the diseased forest in Victoria. Later, the pathogen was recovered from native vegetation in South Australia and from heathland in Queensland (Weste & Marks 1987). The geographic origin of *Ph. cinnamomi* in Australia has long

been the subject of controversy and discussion, particularly in eastern coastal regions. However, it is accepted that the fungus is a relatively recent introduction to Western Australia and probably also to much of southern Australia (Pratt & Wrigley 1970, Weste & Taylor 1971, Pratt *et al.* 1972, 1973, 1974, Pratt & Heather 1972, 1973^a, 1973^b, Podger 1972, Weste *et al.* 1973, Weste & Law 1973, Broadbent & Baker 1974, Weste & Marks 1974).

Ph. cinnamomi has a long history of destruction of Australian forests. In Western Australia, a disease called "jarrah dieback" was recorded as early as 1920 (Podger 1972). *Ph. cinnamomi* was found to be responsible for the disease (Podger *et al.* 1965, Podger 1972). Jarrah is the main timber species in the W.A. state forests, accounting for 75% of the timber cut (Batini 1973). In 1975 the area of jarrah forest affected by dieback was 282000 ha, increasing by 20000 ha per annum (Shea 1975). By 1982 the affected area had escalated to 14% of the state forests (Weste & Marks 1987), resulting in great loss in timber production. Apart from dieback of *E. marginata*, *Ph. cinnamomi* has caused root rot disease on thirty-six species of *Eucalyptus* (Podger & Batini 1970). Many other native plants in the state forest were also affected, including species of *Banksia*, *Macrozamia*, *Podocarpus*, *Casuarina*, *Xanthorrhoea*, and *Isopogon* (Zentmyer 1980). The death of fifty-nine indigenous species in thirty-four genera and thirteen families has been recorded from the infected jarrah forest (Podger 1972). Even the fauna were threatened because of destruction of food sources and nesting site (Christensen 1973). In Victoria, Weste and her colleagues found that most of the 42 tested plant species comprising the woody flora were susceptible to damage by *Ph. cinnamomi*. These included a wide range of plants such as the species of *Pinus*, *Xanthorrhoea*, *Isopogon*, *Hibbertia*, *Pultenaea*, *Banksia*, *Hakea*, *Leucopogon*, *Acacia*, *Grevillea*, and *Epacris*. In diseased areas, *Ph. cinnamomi* attacked most plants within the community, killing 50% of the species. On

severely diseased sites, over 40% of the native stringybarks were killed (Weste 1974, 1975, 1975b, 1977, Weste & Taylor 1971, Weste *et al.* 1973, Weste & Law 1973, Weste & Marks 1974, Weste & Ruppin 1975, 1977, Weste *et al.* 1976, Weste & Vithanage 1977). *Ph. cinnamomi* was also reported to cause severe damage to the flat coastal forests and temperate rainforest in Tasmania (Weste & Marks 1987).

Ph. cinnamomi was the most serious pathogen of avocado and pineapple in subtropical New South Wales and Queensland (Newhook & Podger 1972, Pegg & Alcorn 1972). Following the wet summer of 1974, 50% of all avocado trees were lost from *Phytophthora* root rot, and pineapple suffered 10% reduction from pineapple wilt and top rot in Queensland (Pegg 1977, Zentmyer 1980). In Northern Territory, *Ph. cinnamomi* was isolated from avocados brought from central Queensland, and it spread rapidly to other avocados (Blowes & Pitkethley 1981). An average of 5% of the macadamia trees in Queensland have *Ph. cinnamomi* trunk canker; 50% of these trees have died (Pegg 1973).

Pythium ultimum Trow (*Py. ultimum*) is distributed world-wide as a major plant root pathogen. It was one of the first soil-borne plant pathogens to have been studied in depth (e.g. Hartley 1921 cited by Baker 1987). This fungus is nonspecific as to host plants and affects a large number of plant species (Hendrix & Campbell 1973). Since mature, lignified plant tissues are not affected, whereas juvenile or succulent plant tissues are very susceptible to infection, *Py. ultimum* is especially damaging to plants in the seedling stage (Hendrix & Campbell 1973, Sutherland 1989). At the early seedling stage, *Py. ultimum* commonly infects plant seed and radicle causing seed rot and pre-emergence damping-off. The fungus also infects newly emerged seedlings at ground level, causing post-emergence damping-off. At the later seedling stage, *Py. ultimum* causes plant root rot by infecting young and succulent feeder roots,

leading to poor root development, shoot stunting, and chlorosis, resulting in poor plant growth and yield (Hendrix & Campbell 1973, Sutherland 1989).

Examples of severe diseases caused by *Py. ultimum* resulting in economic losses can be found in such diverse plant types as forest trees, agricultural crops, horticultural crops, and vegetables. For example, root diseases and damping-off caused by *Py. ultimum* were the main disease problems in nurseries where coniferous forest trees were grown for reforestation or afforestation purposes in British Columbia, Canada (Sutherland 1989). Root diseases and damping-off of guayule caused by *Py. ultimum* in nurseries severely damaged the rubber industry in California (Campbell & Sleeth 1945). *Py. ultimum* was the cause of poor emergence and stunting of cotton (Roncadori & McCarter 1972). It was also the common cause of root rot of beans and peas (Kraft & Burke 1971), and various grain crops such as wheat and corn (Hendrix & Campbell 1973). *Py. ultimum* caused root diseases of sugarcane, safflower, soybeans, sweet potatoes, red clover, lettuce and alfalfa (Hendrix 1964, Hendrix & Campbell 1973). It also caused the root disease leading to decline of citrus (Sleeth 1953), peaches, and pears (Hendrix & Campbell 1973).

Since *Ph. cinnamomi* and *Py. ultimum* are soil-borne plant pathogens, they are more difficult than above-ground pathogens to control effectively using fungicides. Furthermore, the application of pesticides has been causing more and more concern world-wide because of residual effects as environmental pollutants as well as the high costs for pesticide development and registration and the development of resistance by the pathogens. Biological control is an alternative disease control method.

In this research, I studied the interactions between plants, plant pathogenic fungi (*Ph. cinnamomi* and *Py. ultimum*), and the potential biological control bacterium (*Pseudomonas cepacia* Burkholder or *Ps. cepacia*). The

associations of such interactions with soil temperature and soil matric potential were also a major component of this study. The overall aim of the study was to explore the possibility of using the bacterium for the control of plant diseases caused by the fungi.

1.2. The association of soil environmental conditions with *Phytophthora cinnamomi* and *Pythium ultimum* and with the diseases they cause.

Phytophthora cinnamomi produces four spore stages (zoosporangia, zoospores, chlamydospores and oospores) in its life cycle (Zentmyer 1980). *Pythium ultimum* produces sporangia, chlamydospores and oospores, but not zoospores (Drechsler 1946, Hendrix & Campbell 1973). *Ph. cinnamomi* is believed to infect plants by mycelium and zoospores (Marx & Bryan 1969, Malajczuk & Theodorou 1979) whereas *Py. ultimum* infects plant by mycelium and sporangial germ tubes. Soil environmental factors play important roles in the growth, reproduction and survival of *Ph. cinnamomi* and *Py. ultimum* and in the disease incidences.

1.2.1. The effects of soil temperature and soil moisture on *Ph. cinnamomi* and *Py. ultimum* and on the diseases they cause.

Of environmental factors, soil temperature and moisture are important in affecting *Ph. cinnamomi* and *Py. ultimum* in their mycelial growth (Sommers *et al.* 1970, Reeves 1975, Gisi *et al.* 1980, Zentmyer 1980, Lifshitz & Hancock 1983, Benson 1984), production, germination and conversion of spores (Bainbridge & Ayers 1970, Sommers *et al.* 1970, Lumsden ~~et al.~~ 1975, Reeves 1975, Gisi *et al.* 1980, Zentmyer 1980, Kuan ^{& Erwin} ~~et al.~~ 1982, Lifshitz & Hancock 1983, Benson 1984), saprophytism and survival (Hoppe 1966, Zentmyer & Mircetich 1966, Munnecke & Moore 1969, Hendrix & Campbell 1973), and consequently the diseases they

cause (Bateman 1959, 1961, Chee & Newhook 1965, Kraft & Roberts 1969, Pieczarka & Abawa 1976, 1978).

Determination of cardinal temperatures for growth on various media of 50 Australian isolates of *Ph. cinnamomi* showed that growth did not occur outside the range 5-35°C (Shepherd & Pratt 1974). Zentmyer reviewed the extensive data on the effect of soil temperature on growth of *Ph. cinnamomi* and stated that the cardinal temperatures for growth were 10°C, 24-27°C, and 32-34°C. However, in the same article, he also gave the cardinal temperatures for growth as 5-15°C, 20-32.5°C, and 30-36°C. The cardinal temperatures for production of sporangia and chlamydospores are similar to that for growth, whereas the optimum temperatures for oospore production are lower. *Ph. cinnamomi* was classified as a moderate temperature species (Zentmyer & Marshall 1959, Zentmyer 1980).

Although the association of soil temperature and moisture with the incidence of diseases caused by *Ph. cinnamomi* and *Py. ultimum* have been studied extensively, the results obtained by different researchers were not always identical. For example, although it has been widely accepted that diseases caused by *Ph. cinnamomi* were more severe in wet than in dry soils (Sterne *et al.* 1976, Sterne *et al.* 1977, Gilmour *et al.* 1979, Wilcox & Mircetich 1979, Kuan & Erwin 1980, 1982, Blake & MacDonald 1981, Matheron & Mircetich 1985), there were also reports that diseases caused by *Ph. cinnamomi* occurred with equal severity in dry and wet soils (Blaker & MacDonald 1981, Matheron & Mircetich 1985).

Soil temperature reflects the balance between the heat energy gains and losses (Armson 1977). Solar radiation provides the primary heat energy which affects the air temperature. Part of the energy is radiated back by the soil surface, part is used in water evaporation, and part is received by the soil resulting in temperature rise.

Soil moisture content exerts a great influence on the soil temperature regime (Armson 1977). Volumetric heat capacity, which is the quantity of heat required to raise the temperature by 1°C of 1 cm^3 soil, is an important thermal property. The volumetric heat capacity of water is higher than that of most soil solids and greatly exceeds that of a unit ~~volumen~~^{Volume} of soil when gases fill the voids. The volumetric heat capacity of a soil therefore increases with increase in moisture content. The rate of temperature change in soil of high water content is therefore less than in dry soil. Another influence of soil moisture originates through the cooling caused by evaporation. The rate of evaporation is largely controlled by differences in water potentials, temperatures and tortuosity of pathways between a source and a sink for water.

Complex interactions therefore exist between air temperature, soil moisture and soil temperature. The temperature regime of a particular soil may be very different at various soil water contents (which is associated with different matric potentials) in one location with a particular range of air temperatures, or at the same water content but at different locations with different air temperatures. Such complex interactions between air temperature, soil moisture and soil temperature are important in determining the disease incidence caused by *Ph. cinnamomi*. It is therefore important that, in the design of experiments and in the interpretation of results, attention must be given to these interactions rather than to a particular variable. It is not surprising that results and conclusions obtained by different researchers are not always identical.

A particular soil environmental factor may play quite different roles in the disease incidences in different pathosystems. Conclusions on the role of soil temperature and moisture cannot be made without taking into account the specific soil and host plant characteristics. Such conclusions should be based on

a systematic study in regard to the effects of soil temperature and moisture on the plant, on various stages of pathogen life cycle and finally on the disease incidence.

Soil temperature and moisture may affect disease incidence through their direct and indirect influences on both the pathogens and the plants. For example, the root disease severity of *Persea indica* was linked to the effect of soil matric potential on *Ph. cinnamomi*. The percentage of diseased roots averaged 80 to 90% at matric potentials of zero; 50% to 90% at -5 kilopascal (kPa); and 10 to 50% at -10 kPa. At -25 kPa, only a few lesions occurred (Sterne *et al.* 1977). However, the soil matric potential may also have directly affected the disease severity by predisposing *Persea indica* plants to the pathogen in saturated soil. In fact, there are many examples of such direct effects on the plants. Jehne (1971) concluded that the primary cause of root rot of *Pinus radiata* caused by *Ph. cinnamomi* was the adverse soil environment, in which waterlogging restricted rooting, stimulated *Ph. cinnamomi* activity and led to stress through subsequent drought because of the inability of the tree roots to maintain sufficient water uptake. Blaker & MacDonald (1981) found that soil moisture extremes (wet or dry) that commonly occur both in nursery and landscape plantings could predispose normally resistant rhododendrons to root and crown rot caused by *Ph. cinnamomi*. It was also found that severe diseases caused by *Py. ultimum* were closely and positively associated with low soil temperature and high soil moisture (Bateman 1959, 1961, Kraft & Roberts 1969, Pieczarka & Abawa 1976, 1978). Soil moisture did not affect disease severity through its direct effects on the pathogen, but through its influence on the amount of plant root exudates. High soil matric potential increased the plant root exudation which provided the fungus with nutrients necessary for germination and growth in soil and thus

increased the disease incidences (Kerr 1964, Schroth and Cook 1964, Stanghellini & Hancock ~~et al.~~ 1971b, Osburn & Schroth 1986).

Similarly, it is also important not to neglect the pathogens when conclusions on the importance of soil temperature and moisture are based on the linkage between their effects on the plant and that on the disease incidences. For example, it was found that resistance of *Banksia grandis* and *Eucalyptus marginata* to *Ph. cinnamomi* changed with soil temperature (Shearer & Shea 1987, Shearer *et al.* 1987). Grant & Byrt (1984) concluded that *Eucalyptus marginata* was susceptible to *Ph. cinnamomi* at 28°C, but resistant at 14°C. However, that the plant was not susceptible at 14°C may be simply because the activity of the pathogen was reduced at that lower temperature.

A plant pathogen may need only a small population in the soil to cause severe plant disease. Soil temperature and moisture may impose significantly adverse effects which reduce the pathogen population but not the disease incidence. It is therefore necessary to study the effects of soil temperature and moisture on the pathogen and relate them to the disease incidence. For example, Nesbitt *et al.* (1979b) found that soil moisture and temperature affected hyphal lysis and sporangial production in *Ph. cinnamomi*. Hyphal lysis was most rapid in soils at, or below, field capacity when incubated at 25-27°C. Sporangia were not formed in soils below field capacity and only very slowly in waterlogged soil. Sporangia were not formed at temperatures below 15°C. Weste *et al.* (1976, ~~1977~~ ^{Weste & Ruppin (1975, 1977)} and ~~1977~~) found that both low temperature from autumn to early summer and dry soils in summer and autumn were correlated with a significant decrease in *Ph. cinnamomi* population, in the Brisbane Ranges independently of site. These results provided valuable information on the range of soil temperature and moisture that may adversely influence the pathogen, but whether such adverse effects were enough to reduce the disease incidence needed further investigation.

Plant fungal pathogens usually produce several morphologically and functionally different stages in their life cycles and soil temperature and moisture may significantly affect pathogens at some life-cycle stages but not at others. The roles of the various pathogen stages in disease incidence and their association with soil temperature and moisture need to be studied. Although *Ph. cinnamomi* may infect plants by mycelium and zoospores, the role of mycelium in plant infection and its association with soil temperature and moisture have not received enough attention (Marx & Bryan 1969, Malajczuk & Theodorou 1979). Enhanced sporangial production and zoospore release were usually said to lead to severe diseases in wet soils (Zentmyer 1980).

Experiments on the associations of soil temperatures and matric potentials with turnip seedling growth and with the damping-off of turnip caused by *Ph. cinnamomi* and *Py. ultimum* are reported in chapter 3. The associations of soil matric potentials with the mycelial growth and reproduction of *Ph. cinnamomi* and with the mycelial growth of *Py. ultimum* were studied (Chapter 6). By linking and discussing the results, I attempt to provide an overview in regard to the role of the plant and that of the fungal growth and reproduction in the disease incidences and their associations with soil temperature and matric potential used. The interaction between soil temperature and soil matric potential is also discussed.

1.2.2. The association of nutrients with *Ph. cinnamomi* and *Py. ultimum* and with the diseases they cause --- A literature review.

Nutrient availability is another important factor which affects *Ph. cinnamomi* and *Py. ultimum* and the diseases they cause. In experiments reported in this thesis, I did not study the effects of nutrients on *Ph. cinnamomi* and *Py. ultimum* disease incidences. However, the importance of nutrients in

the interactions between *Ps. cepacia* and the fungi was a major part of the studies.

Ph. cinnamomi showed great variation in growth and spore production depending on nutrient source. Roncadori (1965) studied the nutritional requirements of 60 isolates belonging to 25 species of *Phytophthora*. He found that glucose, fructose, mannose, and xylose were good carbon sources for growth of *Ph. cinnamomi* and that KNO_3 was a good nitrogen source. Chee and Newhook (1965) tested 20 carbon sources for growth and sporulation of *Ph. cinnamomi* in soil. The best carbon sources for growth and sporangial production were dextrin, starch, and sucrose. Growth was slow with xylose, but many sporangia were produced. Ayer and Zentmyer (1971) found that production of sporangia by *Ph. cinnamomi* was inhibited by glucose and glutamic acid under axenic conditions and in nonsterile soil extracts.

Chlamydospore germination in *Ph. cinnamomi* and *Py. ultimum* was also influenced by nutrients. The chlamydospores of *Ph. cinnamomi* germinated either by numerous germ tubes or by a germ tube bearing a sporangium, depending on the level of available exogenous nutrients (Mircetich *et al.* 1968). In nonamended, natural soils, large numbers of germinated chlamydospores developed a short germ tube bearing a sporangium, whereas in glucose- or asparagine-amended soils most germinated chlamydospores developed several germ tubes that formed mycelia (Mircetich & Zentmyer 1969). Low amounts of available nutrients in the natural soil were thus found to be the primary factor limiting germination (Mircetich *et al.* 1968, Mircetich & Zentmyer 1969). Sporangia of *Py. ultimum* stored in soil for 11 months germinated in 1.5 h and germination reached a maximum 3 h after nutrients were added to soil (Stanghellini & Hancock 1971). More germination of sporangia of *Pythium* was induced and lysis was prevented when soil was amended with glucose, maltose

and particularly sucrose or with glutamic acid, yeast extract, and asparagine (Agnihotri & Vaartaja 1967). Survival of *Py. ultimum* was affected by soil nutrition. Sterols and calcium stimulated oospore formation, leading to greater survival (Hendrix 1964, Yang & Mitchell 1965).

Only a few studies examined the vitamin requirements of *Ph. cinnamomi*. However, it was confirmed that thiamine was the only vitamin required by the fungus (Chee & Newhook 1965, Cameron 1966, Roncadori 1965).

Nutrition also affected *Ph. cinnamomi* and *Py. ultimum* disease incidences. For example, nitrate and potassium reduced damping-off of tomato and cucumber caused by *Py. ultimum* in moist soil, whereas ammonium and phosphate did not (Yale & Vaughan 1962). Deficient or excessive levels of calcium increased susceptibility of bent grass to *Py. ultimum* (Hendrix & Campbell 1973). It was also suggested that at -25 kPa in sandy loam, soil nutrient availability rather than matric potential limited root disease caused by *Ph. cinnamomi* (Sterne *et al.* 1977).

1.3. Biological control by *Pseudomonas cepacia* of plant diseases caused by *Phytophthora cinnamomi* and *Pythium ultimum*.

1.3.1. Biological control of plant diseases --- General concepts.

Within the natural soil which is inhabited by a mixed microbial population, antagonism is a commonly occurring phenomenon between the microorganisms. All microorganisms are affected, directly or indirectly, by one or more other microorganisms (Garrett 1950). Antagonism refers to a reduction in growth and in activity of one microorganism, as a result of the activity of one or more other microorganisms (Waksman 1941). Antagonism can be divided into "general antagonism" and "specific antagonism" (Gerlagh 1968). General antagonism refers to the antagonism experienced by one organism as a result of

the activity of a wide range of organisms, while specific antagonism refers to the antagonism against one organism by a single organism or a specific group of organisms.

Variation in disease incidence in natural environments has long been recognized (Cook & Baker 1983). Naturally occurring plant disease suppression has been recognized since 1892 when *Fusarium* wilt diseases of cotton were found to be more prevalent in sandy soils than in clay (Baker 1987). Biological control was defined by Baker (1987) as "the decrease of inoculum or the disease-producing activity of a pathogen accomplished through one or more organisms, including the host plant but excluding man". It is the antagonism between plant pathogens and other microorganisms that form the basis for the development of biological control of plant disease.

Biological control of plant disease can be achieved by modification of the biological equilibrium of the pathosystem in ways that are unfavorable to the plant pathogens but favorable to their natural antagonists.

Suppressive soil was defined as a soil in which disease development was suppressed even though the pathogen was introduced in the presence of a susceptible host plant (Baker & Cook 1974, Hornby 1983). On the other hand, soil in which disease development was stimulated was defined as conducive soil. Although physical and chemical environmental factors may be responsible for disease suppression, most examples of suppression in soil were biological in nature (Scher & Baker 1980, Baker 1987). Suppressive soils are of great importance in the biological control of plant disease and can be exploited in many ways. Monoculture is sometimes a method for the induction of suppressiveness. For example, specific suppression of take-all was fostered by wheat monoculture but may be lost from a soil by fallow or rotation with certain crops (Cook & Rovira 1976). Disease suppression can also be obtained by

transferring a portion of suppressive soil into a conducive soil. Suppressive soils are good ~~resources~~ sources for the isolation of promising biological control agents of plant diseases.

The biological balance of the pathosystem can also be directly modified by additions of inorganic or organic matter~~s~~, called "soil amendment~~s~~". Organic soil amendment is of great importance in the biological control of plant disease, because the addition of already decomposed amendments may introduce into the pathosystem new microorganisms which are adapted to the ~~addition~~ ^{amendment} and can compete with the pathogen more effectively. Soil suppressiveness can be enhanced by soil amendments, and transferred to disease conducive soils (Boosalis 1956, Cook & Rovira 1976).

The biological balance of the pathosystem can be indirectly modified through the physical characteristics of the soil. For example, soil pH can be affected by either liming or the addition of a fertilizer. Soil water can be controlled by irrigation, drainage and tillage. Tillage can also affect soil temperature to some extent.

Biological control of plant disease can also be achieved by artificially introducing into the pathosystem a microorganism or a group of microorganisms selected for their potential antagonism to the pathogen (Papavizas & Lumsden 1980). Many soil microorganisms, including fungi, actinomycetes and bacteria, are antagonistic to a range of plant pathogens and thus can be explored for the control of plant diseases (Wood & Tveit 1955, Cooksey & Moore 1982, Capper & Campbell 1986).

Biological control of plant disease by antagonists occurs through various mechanisms. Theoretically and traditionally, these mechanisms are classified as hyperparasitism and predation, antibiosis, lysis and competition.

Hyperparasitism occurs when one microorganism is parasitic on another (Boosalis 1964). It involves an intimate contact between the host fungus and the hyperparasite. Mycoparasitism is a well-documented phenomenon referring to the parasitism of one fungus by another (Barnett & Lilly 1962, Griffith & Barnett 1967, Barnett & Binder 1973). Mycoparasites are widespread and examples can be found among all groups of fungi from the chytrids to the higher basidiomycetes (Durrell 1968, Huang 1977, 1978, Ayers & Adams 1979, 1979, 1981, Lumsden 1980). Bacterial parasitism is similar in concept, a bacterium parasitic on another microorganism.

Antibiosis refers to the inhibition of one microorganism by another through the production of antibiotics, i.e. antagonistic metabolites. Mycolysis is defined as the loss of protoplasm in fungal structures and the dissolution of the cell wall due to the enzymatic action by other microorganisms (Lloyd & Lockwood 1966). The capacity to produce cell wall-degrading enzyme is common among soil bacteria and saprophytic fungi (Papavizas 1985).

Soil microorganisms compete with each other for infection sites and nutrients. Competition for the infection site is influenced by the law of priority. A microorganism already in a substrate may retain possession against vigorous competitors by various means including converting nutrients into a form unavailable to other competitors, producing antibiotics, and preventing the accumulation in the infection site of excess nutrients which would support other competitors. Plant cross protection is a term used to describe the ability of a microorganism to protect a plant from a pathogen without producing any inhibitory substance (Ricard 1977). Although the mechanisms for plant cross protection by fungi have not yet been fully understood, competition for infection sites is often involved (Fletcher 1978, Ogawa & Komada 1984). For example, it has been suggested that niche exclusion is potentially an important mechanism of

antagonism against deleterious rhizobacteria by plant growth-promoting rhizobacteria (Suslow 1982, Suslow & Schroth 1982).

Competition on plant roots for nutrients present in root and seed exudates occurred in most interactions between bacteria and pathogens and was responsible for the observed biological control by some introduced bacteria (Suslow 1982, Elad & Baker 1985, ^{Elad & Baker 1985b,} Elad & Chet 1987). Large populations of bacteria established on plant material and roots become a partial sink for nutrients in the rhizosphere, thus reducing the amount of carbon and nitrogen available to stimulate spores of fungal pathogens or for subsequent colonization of the root (Elad & Baker 1985, ^{Elad & Baker 1985b,} Elad & Chet 1987).

Ferric ions (Fe^{3+}) play a central role in the energy metabolism of aerobic and semi-anaerobic microorganisms (Schippers 1988). It is essential for fungal spore germination (Kloepper *et al.* 1980, ^{Elad & Baker 1985b,} Smiley 1980, ~~Vanderberg *et al.* 1983,~~ ^{Kloepper *et al.* 1980b, Kloepper *et al.* 1980} Sneh *et al.* 1984). Its availability in soil for microorganisms and plants decreases dramatically as pH increases above 6 (Schippers 1988). When the availability of Fe^{3+} in soil is limited, competition for it between soil microorganisms is inevitable and therefore may result in effective disease control (Kloepper *et al.* 1980, Neilands 1981, Teintze *et al.* 1981, Ahl *et al.* 1986, Baker *et al.* 1986, Bakker *et al.* 1986, ^{Kloepper *et al.* 1980b, Kloepper *et al.* 1980} Leong 1986, Schippers 1988).

1.3.2. The biological control of diseases caused by *Phytophthora* spp. and *Pythium* spp., and *Ps. cepacia* as a biological control agent---A literature review.

Many possible approaches to biological control of plant diseases caused by *Phytophthora* spp. and *Pythium* spp. have been reported in the past.

Soils suppressive to *Phytophthora cinnamomi* in Queensland were shown to be effective in controlling avocado root rot. The addition of *Ph. cinnamomi* inoculum in amounts sufficient to cause severe root rot of plants in other soils produced little or no damage in the suppressive soil. The suppressive soil was

found to have a higher population of bacteria and actinomycetes than soils conducive to root rot. These suppressive soils became conducive when steamed at 100°C for 30 min, but remained suppressive after steam treatment at 60°C for 30 min (Broadbent *et al.* 1971, Pegg & Alcorn 1972, Pegg 1973, 1977, Broadbent & Baker 1974, Broadbent *et al.* 1977, Baker 1987).

Disease-suppressive soils are attractive resources for obtaining potential microorganisms to control plant diseases. For example, Harman *et al.* (1980) obtained biological control against *Pythium* through seed treatment with *Trichoderma hamatum* isolated from soil observed to be suppressive to *Rhizoctonia solani*. Tomato seed inoculation with *Arthrobacter* spp. protected the young seedlings against damping-off caused by *Pythium debaryanum* in aseptic culture and in non-sterile soil (Mitchell & Hurwitz 1965).

Soil amendment is another effective approach to the control of *Ph. cinnamomi* and *Py. ultimum* diseases (Hoitink 1986). *Ph. cinnamomi* propagules that normally cause severe root rot of lupin seedlings produced little damage to similar seedlings growing in hardwood bark-sand compost (Hoitink *et al.* 1977). A significant inhibition of sporangial production and lysis of zoospore and cysts occurred in the bark compost. Such suppressive effect of the bark compost was due to chemical and biological rather than physical factors (Hoitink *et al.* 1977). Satisfactory control of *Phytophthora* root rot of avocado was also achieved by using a cotton waste amendment which increased the population of microorganisms in the *Phytophthora* infested soil (Gilpatrick 1969). Alfalfa meal mixed with soil at rates of 1-5% gave good control of *Phytophthora* root rot of avocado seedlings as well as root rot and stem canker of *Persea indica* (Zentmyer 1963). The great increase in microbial population after addition of alfalfa meal was a factor in the biological control of *Phytophthora* root rot. A high degree of control of strawberry red stele caused by *Phytophthora*

fragariae was achieved by a composted Douglas fir sawdust (Vaughn *et al.* 1954). Compost amendment also improved suppression of damping-off caused by *Rhizoctonia solani* and *Py. ultimum* because a combination of fungal and bacterial antagonists were introduced in peat media (Kuter & Hoitink 1985). The effect of composted sewage sludge on a wide range of diseases was examined in glasshouse trials (Lumsden *et al.* 1983). Incorporation into soil of composted municipal sludge controlled *Phytophthora* crown rot of pepper. *Pythium* damping-off of pea was not suppressed initially, but the treated soil became suppressive with time.

Diseases caused by *Phytophthora* spp. and *Pythium* spp. may be controlled by artificially introducing into the pathosystem fungi and bacteria antagonistic to the pathogens. Bacterial antagonists have been reported from many genera. For example, biological control of apple and cherry crown and root rot caused by *Ph. cactorum* were obtained by applying a siderophore-producing *Pseudomonas* sp. (Janisiewicz and Covey 1981) and *Bacillus subtilis* (Utkhede 1984a, 1984b, 1985, 1986, 1987, Gupta & Utkhede 1986). Several *Streptomyces* spp. reduced root rot of soybean caused by *Ph. megasporum* when added to soil at the same time or 7 days prior to addition of the pathogen (Rothrock & Gottlieb 1981). Wheat (Weller & Cook 1981, 1983, 1986, Weller 1988), cotton (Howell & Stipanovic 1979, 1980, 1983), and sugar beet (Osburn *et al.* 1989) were protected against *Pythium* spp. by *Pseudomonas* spp.

Fungal biological control agents such as species of *Trichoderma* and *Gliocladium* have been reported to produce antibiotics which were toxic to a wide range of fungal plant pathogens (Papavizas 1985). Hartley made the first attempt in 1921 at direct application of biological control against damping-off of pine seedlings caused by *Py. ultimum*, by inoculating soil with a range of fungal antagonists (Baker 1987). In 1932, while studying the damping-off of citrus

seedlings, Weindling found a strain of *Trichoderma lignorum* that parasitized *Ph. parasitica* and species of *Pythium*. Later (1941), he illustrated hyphae internally invaded by *Trichoderma*. The studies were, however, conducted only *in vitro*. Microscopic observations of soil microorganisms in soil indicated that parasitic microorganisms aided the decline of population of *Py. ultimum* in soil (Hancock 1981). *Gliocladium virens* effectively controlled damping-off of zinnia, cotton, and cabbage caused by *Py. ultimum* or *Rhizoctonia solani* in a nonsterile commercial soilless mix (Lumsden & Locke 1989). When *G. virens* was grown on a bran extract or a minimal medium supplemented with dead *Py. ultimum* hyphae, aqueous extracts from the cultures contained gliotoxin, laminarinase, amylase, carboxymethylcellulase, chitinase and protease and inhibited sporangial germination and mycelial growth of *Py. ultimum* (Roberts & Lumsden 1990). Sneh *et al.* (1977) studied the invasion of oospores of *Ph. megasperma* var. *sojae*, *Ph. cactorum* and *Pythium* sp. in soil. They discovered that an array of microorganisms, including oomycetes, chytridiomycetes and hyphomycetes fungi, and actinomycetes and bacteria, were capable of parasitically invading and destroying the oospores (Sneh *et al.* 1977). Several isolates of *Ph. cinnamomi* were similarly sensitive to inhibition caused by the ectotrophic mycorrhizal fungus *Leucopaxillus cerealis* var. *piceina* (Marx 1969, 1969). Culture filtrates of this fungus reduced the growth of *Ph. cinnamomi* and completely inhibited zoospore germination. Ectomycorrhizae formed by several fungi on roots of *Pinus radiata* reduced or completely prevented infection by mycelium and zoospores of *Ph. cinnamomi* (Marx 1969^a, 1969^b). *Mortierella* spp. isolated from azalea and boxwood roots were inhibitory in *in vitro* studies to growth of *Ph. cinnamomi* pathogenic to azalea and *P. parasitica* pathogenic to boxwood. They also protected azalea cuttings from *Ph. cinnamomi* infection (Willis & Lambe 1980). A bran/peat preparation of a *Penicillium funiculosum*

isolate which showed antagonism to several *Phytophthora* spp. *in vitro* suppressed azalea root rot caused by *Ph. cinnamomi* or *P. parasitica* in glasshouse tests (Fang & Tsao 1989). A strain of *Myrothecium roridum* was isolated from the rhizosphere soil of avocado roots grown in a soil suppressive to *Ph. cinnamomi* (Gees & Coffey 1988, 1989). Application of a wheat bran preparation of the fungus at a rate of 2.5% (W/V) in glasshouse and in field tests effectively controlled the seedling disease of *Persea indica* caused by *Ph. cinnamomi*. In a glasshouse, the biocontrol was comparable to the control achieved by application of potassium phosphonate at 2.5 mg/pot (Gees & Coffey 1988, 1989).

In recent years, studies of *Ps. cepacia* as a biological control agent of plant diseases have been increasing. *Ps. cepacia* significantly increased plant fresh weight of China aster in the presence of *Fusarium* in field test (Cavileer & Peterson 1985). Protection of onion seedlings from damping-off caused by *Fusarium oxysporum* f. sp. *cepae* was obtained by seed and soil infestation with *Ps. cepacia* (Kawamoto & Korbeer 1976). A study on the distribution of *Ps. cepacia* in North Carolina, USA, showed that all soil and root samples studied contained *Ps. cepacia*. Several strains were antagonistic to one or more tobacco pathogens (Spurr & Sasser 1982). *Ps. cepacia* was antagonistic to *Pythium aphanidermatum* and protected cucumber seedlings from infections by the fungus in natural soil (Lumsden *et al.* 1982). Several *Ps. cepacia* strains were able to colonize maize roots grown in non-sterile soil even at very low inoculum levels (Hebbar 1986). *Ps. cepacia* also reduced maize seedling infection by *Fusarium moniliforme* by as much as 50% to 80% (Hebbar 1986). *Ps. cepacia* strain 526 was found to produce three antibiotics, pyrrolnitrin, aminopyrrolnitrin and xylocandin (Hebbar 1986, Dr P.J. Dart & Mr R. Rickards, personal communications). Parker *et al.* (1984) found that strains of *Ps. cepacia*

produced two acetylenic antibiotics, cepacins A and B, in fermentation broth. A soil isolate of *Ps. cepacia* also showed strong antibiotic activity against *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium irregulare* and *Laetisaria arvalis* in culture tests (Conway *et al.* 1989).

1.3.3. Biological control by *Ps. cepacia* of diseases caused by *Ph. cinnamomi* and *Py. ultimum* and associations with soil environmental conditions--- Justifications.

Although diseases caused by *Ph. cinnamomi* and *Py. ultimum* have been major targets for biological control studies and *Ps. cepacia* has been found to be a potential biological control bacterium, there are no reports regarding biological control by *Ps. cepacia* of diseases caused by either *Ph. cinnamomi* or *Py. ultimum*. In experiments of this thesis, I studied the interactions between *Ps. cepacia* strain 526 and the fungi and the biological control effects of the bacterium on ~~the turnip~~ damping-off of turnip caused by the fungi.

In a soil pathosystem involving plant, plant pathogen and other soil microorganisms, various complex interactions occur between plant and plant pathogen, between plant and soil microorganisms, and between plant pathogen and soil microorganisms. These interactions are again affected by the complex soil environmental conditions. Many soil environmental factors are involved in determining the plant disease incidences. These soil environmental factors have multifaceted effects, on the host, making it more or less susceptible; on the pathogen, making it more or less virulent; and on the soil microflora, which in turn affect the host plant and the pathogen. Through their combined effects they determine the disease incidence. Interactions between several soil environmental factors are commonplace. The successful application of a microorganism as a disease biological control agent will largely depend on our understanding of such complex interactions through ecological studies.

Although studies of biological control of plant disease have a history of more than 70 years, only a few microorganisms have been commercialized and successfully used (Rishbeth 1963, 1975, Kerr 1972, 1980, 1982, Deacon 1988). This is largely due to the lack of ecological studies (Deacon 1988).

Soil temperature (Gregory *et al.* 1952, Dunleavy 1955), moisture (Griffin 1963^a, 1963^b, 1966^a, 1966^b, 1969, 1972, 1977, 1978, 1981^a, 1985, Wong & Griffin 1974, 1976^a, 1976^b, Luard & Griffin 1981, Howie *et al.* 1987) and nutrients are important factors in affecting the growth, movement, reproduction, and antagonistic effects of introduced microorganisms and in determining their disease biological control effects. For example, biological control effect by *Ps. cepacia* was affected by soil temperature, moisture and nutrients. *Ps. cepacia* antagonistic *in vitro* to *Pythium aphanidermatum* protected cucumber seedlings from infection by the fungus in natural soil. The addition of a nutrient solution increased the population of *Ps. cepacia* in a sandy loam soil. *Ps. cepacia* survival in soil was better at 20°C than at 30°C, and at 0.3 bar (-30 kPa) than at 5 bars (-500 kPa) (Lumsden *et al.* 1982). A *Ps. fluorescens* isolate produced an antifungal compound inhibitory to *Py. ultimum* (James & Gutterson 1986, Gutterson *et al.* 1986, Gutterson *et al.* 1988). Synthesis of the compound was regulated by glucose (James & Gutterson 1986) and depended on expression of at least five genes (Gutterson *et al.* 1986, Gutterson *et al.* 1988). The biological control of *Py. ultimum*-induced damping-off of cucumber and of other crops by *Pseudomonas* sp. has been attributed to competition for carbon and nitrogen (Elad & Chet 1987).

Soil temperature and moisture (refer to 1.2.1) and nutrients (refer to 1.2.2) have critical effects on *Ph. cinnamomi* and *Py. ultimum* and on the diseases they cause. They may affect the *Ps. cepacia* and its antagonistic effects. In experiments reported in this thesis, these environmental variables were studied in relation to the interactions between the fungi and the bacterium (Chapters 4, 5

& 6), and with the biological control by *Ps. cepacia* of diseases caused by *Ph. cinnamomi* and *Py. ultimum* (Chapter 3).

Both *Ph. cinnamomi* and *Py. ultimum* produce several morphologically and functionally different stages in their life cycle (refer to 1.2). *Ps. cepacia* may play important roles in the growth, production and conversion of spores, saprophytism and survival of *Ph. cinnamomi* and *Py. ultimum* and, consequently in the diseases they cause. The biological control by *Ps. cepacia* may have resulted from its antagonism either to a specific stage or to the whole life cycle of the pathogens. It is thus important to understand the roles of *Ps. cepacia* in relation to the life cycle of the pathogens and relate them to disease control. It is also important to understand the associations of such roles of *Ps. cepacia* with the soil environmental conditions so that a successful strategy for its application can be developed.

In this thesis, I report the effects of *Ps. cepacia* 526 on *Ph. cinnamomi* mycelial growth, sporangial production and zoospore release, and on *Py. ultimum* mycelial growth in culture (Chapters 4, 5, 6) and in soil (Chapter 6). The association of nutrient with the effects of *Ps. cepacia* 526 were also studied (Chapters 4 & 5). The association of such effects with soil matric potential are specially reported in chapter 6. Considering all the results, I then discuss the mechanisms by which *Ps. cepacia* affect *Ph. cinnamomi* and *Py. ultimum* growth and reproduction and the diseases.

In selecting biological control agents of soil-borne plant pathogens, the *in vitro* culture test is generally treated as an important step as it is a simple, quick and easy method of screening microorganisms. However, the results of the tests are not always in accord with performance of biological control agents in practice (Utkhede 1986). This is to a large extent due to the great differences between the complex changing soil condition and the relatively uniform and

stable culture condition, especially the nutrient condition. Even culture tests using different media or different methods may give different results, as the quality and the quantity of the nutrients in various media are different. It is therefore important that caution be taken in performing the culture test and in interpreting the results. The results would be more conclusive if *in vitro* tests were performed under various conditions, such as different media differing in their nutrient components, and in different ways, such as with agar and liquid media. The *in vitro* experiments reported in this thesis (Chapters 4 & 5) were based on these principles.

Soil microorganisms may produce different antibiotics which are specifically active against various pathogens. For example, a *Gliocladium virens* isolate produced gliovirin active against *Py. ultimum* but not against *Rhizoctonia solani*. The isolate protected cotton seedlings against damping-off caused by *Py. ultimum* but not that by *R. solani* (Howell 1982, Stipanovic & Howell 1982, ~~1983~~, ^{Howell & Stipanovic 1983}). Purified antibiotics were isolated from a *Ps. fluorescens* culture and identified as pyrrolnitrin (Howell & Stipanovic 1979) and pyoluteorin (Howell & Stipanovic 1980). Pyrrolnitrin was found to be strongly inhibitory to *R. solani*, but not to *Py. ultimum*; whereas pyoluteorin was found to be inhibitory to *Py. ultimum*, but not to *Rhizoctonia solani*. Pyrrolnitrin is not readily diffusible and is released only upon bacterial cell lysis, but is persistent in the soil (Howell & Stipanovic 1979). Pyoluteorin was absorbed and inactivated by the soil colloids when added directly to soil. These antibiotics provided the same protection to cotton against damping-off caused by *Py. ultimum* (Howell & Stipanovic 1980) or *Rhizoctonia solani* (Howell & Stipanovic 1979) as did the bacterium. Pyrrolnitrin was first isolated from a *Pseudomonas* sp. in Japan and was found to be inhibitory to fungi, yeasts and Gram-positive bacteria (Arima *et al.* 1965). It was later isolated from *Ps. aureofaciens* (Gordee & Matthews 1967), and from *Ps.*

cepacia colonizing the bean rhizosphere and appeared to be involved in the biological control of *Py. aphanidermatum* (Lumsden *et al.* 1979). *Ps. cepacia* 526 was also found to produce pyrrolnitrin, aminopyrrolnitrin and xylocandin (Hebbar 1986, Dr. P.J. Dart & Mr. R. Rickards, personal communications).

In this research, I studied and discussed possible variation of *Ps. cepacia* 526 in its antagonistic effects against *Ph. cinnamomi* and *Py. ultimum*. The effects of pyrrolnitrin on *Ph. cinnamomi* and *Py. ultimum* were examined.

The interaction between a plant pathogen and a biological control agent can be mutual. While the biological control agent has antagonistic effects against the plant pathogen, the plant pathogen may also impose a countereffect on the biological control agent. In the studies of such complex interactions, however, the antagonistic effects of the biological control agent on the plant pathogen are usually the focus, but the effects of the plant pathogen on the biological control agent are usually neglected. To achieve successful disease biological control, these may be aspects that require equal attention.

In this thesis, I specifically report the effects of *Ph. cinnamomi* and *Py. ultimum* on *Ps. cepacia* 526 and the associations of these effects with nutrients (Chapters 4 & 5). The importance of such effects in the disease biological controls by *Ps. cepacia* 526 are discussed.

1.4. Rhizosphere effects of plant and the plant growth-promoting effects caused by *Pseudomonas cepacia*.

Soil microorganisms are greatly influenced by plants. It has long been noted that the number and activity of microorganisms are greater in soils closely adjacent to plant roots. The 'rhizosphere' refers to the region in the soil where the soil microorganisms are influenced by the plant roots (Rovira 1965, Rovira & Davey 1974). The rhizosphere is mediated to a large extent by the occurrence of

plant root exudation. Sugars, nitrogen compounds, organic acids and vitamins are major groups of nutrient compounds existing in the root exudates which are readily available for the utilization of soil microorganisms (Schroth & Cook 1964, Rovira 1965, Cameron 1966, Rovira *et al.* 1979, Schonwitz & Ziegler 1982). The soil microorganisms are directly affected by plant root exudation in their growth, spore germination and reproduction and survival. The microorganisms are also indirectly affected by competition and antibiosis by other microorganisms whose activities are affected by plant root exudates.

While plants affect soil microorganisms, soil microorganisms also affect plants. Some bacteria may promote plant growth and are particularly referred as plant growth-promoting rhizobacteria (PGPR) (Burr *et al.* 1978, Kloepper *et al.* 1980, Teintze *et al.* 1981).

Kloepper et al. 1980b, Kloepper et al. 1980
PGPR may directly promote plant growth by production of plant growth hormones which are important in many plant growth processes. For example, inoculation of plants with various bacteria resulted in increased plant growth which could be mimicked by addition of plant growth hormones (Brown 1972, 1974). PGPR may directly promote plant growth also by improving plant nutrient uptake. Some bacteria such as *Bacillus megaterium* and *Ps. fluorescens* were able to decompose organic phosphates into inorganic phosphates and make them usable by the plants (Brown 1974).

PGPR may indirectly change the rhizosphere microbial population (Burr *et al.* 1978, Smiley 1980, Neilands 1981, Teintze *et al.* 1981, ~~Vanderberg *et al.* 1982~~, Bakker *et al.* 1986^b, Leong 1986, Schippers 1988). There are microorganisms in soils which are detrimental to plants but do not cause obvious disease symptoms. These were once called 'minor pathogens'. PGPR may suppress the population of such 'minor pathogens' and thus increase plant growth (Bakker *et al.* 1986^a, Kloepper *et al.* 1987, Schippers 1988). PGPR may also suppress the population of

plant pathogen leading to disease control (Kloepper ^{1980a} *et al.* 1980, Scher & Baker 1982, Weller 1983, 1984, Becker & Cook 1984, Colyer & Mount 1984, Sneh *et al.* 1984, Elad & Baker 1985, Geels *et al.* 1986, Yuen & Schroth 1986^b).

As a biological control agent, the ability of a microorganism to colonize and establish a high population in the rhizosphere soil and on plant roots is important for its effectiveness. Colonization is determined by the complex interactions between the biological agent and the general soil microbiota. Microorganisms which can efficiently compete and utilize the substrates exuded by the plant root usually develop a larger population in the rhizosphere soil and on the plant root than others (Rovira 1965, Bowen & Rovira 1976). The ability to promote plant growth is also an advantage in a biological control agent. Information concerning such abilities of a biological control agent are important for the strategic development of the successful application measures.

There are a few reports of *Ps. cepacia* as a biological control agent (refer to 1.3.3), but information on its ability to colonize the rhizosphere soil and plant roots are rare. Hebbar (1986) found *Ps. cepacia* strains to be good colonizers of maize roots, even when the maize seeds were coated in suspensions with an initial inoculum population as low as 10^1 cfu/ml.

Information on the ability of *Ps. cepacia* to promote plant growth is also inadequate. The growth-promoting effects of *Ps. cepacia* may be affected by the soil environment conditions but no evidence is available. Cavileer & Peterson (1985) found that *Ps. cepacia* significantly increased plant fresh weight of China aster in the presence of *Fusarium* in field test.

In chapter 3 of this thesis, I report the growth-promoting effect of *Ps. cepacia* 526 on turnip seedlings and its association with soil temperature and matric potential. The competitiveness and colonization ability of *Ps. cepacia* 526

in the rhizosphere soil and on plant roots and its growth promoting effects were further studied (Chapter 7).

1.5. Conclusion and thesis outline.

The successful utilization of a microorganism for plant disease control requires a systematic study to obtain understanding of the complex interactions between plant and plant pathogen, between plant and the potential biological control agent and between plant pathogen and the potential biological control agent. Understanding of the associations between these complex interactions and the soil environmental conditions are critical.

The overall aim of the studies described in this thesis was to explore the possibility of using *Ps. cepacia* for the control of plant diseases caused by *Ph. cinnamomi* and *Py. ultimum*. To fulfil the aim, I first examined the antagonisms of *Ps. cepacia* strains against *Ph. cinnamomi* and *Py. ultimum*, and the pathogenicity of the fungi, through a series of preliminary experiments reported in chapter 2, in which an experimental system was established. The pathogenicities of *Ph. cinnamomi* and *Py. ultimum* on turnip, and the plant growth-promoting and the disease biological control effects of a strain of *Ps. cepacia* at various soil matric potentials and temperatures were then studied (Chapter 3). In chapters 4, 5 and 6, the interactions between *Ps. cepacia* and the fungi *Ph. cinnamomi* and *Py. ultimum*, and their association with culture nutrient level and soil matric potentials were reported. The colonizing ability of *Ps. cepacia* in rhizosphere soil and on plant roots and its growth promoting effects were the subjects of chapter 7.

CHAPTER 2. GENERAL MATERIALS AND METHODS

The general materials and methods used throughout the study are described in this chapter. However, materials and methods related to specific experiments are described in the later relevant chapters.

2.1. Culture media.

All agar media were poured into 9 cm sterile plastic petri plates in a laminar flow cabinet. Petri plates were stored at 4°C in plastic bags.

Media for *Phytophthora* spp. and *Pythium* spp.

2.1.1. Potato dextrose agar (PDA).

Dehydrated PDA (Difco) 30 g; distilled water 1000 ml; autoclave at 121°C for 30 min.

2.1.2. Corn meal agar.

Corn meal agar (Difco) 17 g; distilled water 1000 ml; autoclave at 121°C for 30 min.

2.1.3. V-8 agar.

Cleared V-8 juice 100 ml; β -sitosterol 0.02 g; CaCO_3 0.1 g; Bacto agar (Difco) 17 g; distilled water 900 ml; pH was adjusted to 6.0-6.5 with 1N NaOH; autoclave at 121°C for 30 min.

Cleared V-8 juice was obtained by centrifuging canned V-8 vegetable juice at 5000 rpm for 20 min, supernatant filtered through Whatman GF/A. Cleared V-8 juice was autoclaved and stored in a freezer.

2.1.4. Oatmeal agar.

Dehydrated oatmeal agar (Difco) 72.5 g; distilled water 1000 ml; autoclave at 121°C for 15 min.

2.1.5. Czapek Dox agar.

Dehydrated Czapek Dox agar (BBL) 50 g; distilled water 1000 ml; mix until a uniform suspension is obtained; heat with frequent agitation; autoclave at 121°C for 15 min.

2.1.6. Water agar.

Agar 20 g; distilled water 1000 ml; autoclave at 121°C for 30 min.

2.1.7. Selective medium (P₁₀VP; Tsao 1969, Tsao 1970).

Corn meal agar (Difco) 17 g; distilled water 1000 ml; autoclave at 121°C for 30 min. When the medium has cooled down to 40-45°C, add pimaricin (10 ppm), vancomycin (200 ppm) and pentachloronitrobenzene (PCNB, 100 ppm) and shake to dissolve before pouring into petri plates; final pH 6.0.

Media for *Pseudomonas cepacia*.

2.1.8. Nutrient agar.

Nutrient broth (Difco) 8 g; Bacto agar (Difco) 15 g; distilled water 1000 ml; pH 6.8-7.0; autoclave at 121°C for 30 min.

2.1.9. Nutrient broth.

Nutrient broth (Difco) 8 g; distilled water 1000 ml; dispense into conical flasks; autoclave at 121°C for 30 min; store at room temperature .

2.1.10. Selective media.

2.1.10.1. Rifampicin nutrient agar.

Nutrient broth (Difco) 8 g; Bacto agar (Difco) 15 g; distilled water 1000 ml; pH 6.8-7.0; autoclave at 121°C for 30 min; when the medium has cooled down to 40-45°C, add 100 mg rifampicin and 150 mg cyclohexamide and shake to dissolve

before pouring into petri plates. *This is a selective medium commonly used for *Pseudomonas cepacia* which is resistant to the antibiotics at the levels used.*

2.1.10.2. PCAT medium (Burbage and Sasser, 1982).

Azelaic acid 2.0 g; tryptamine 0.2 g; MgSO₄ 0.1 g; K₂HPO₄ 4.0 g; KH₂PO₄ 4.0 g; yeast extract (Difco) 0.02 g; Bacto agar 15.0 g; distilled water 1000 ml.

Add azelaic acid and MgSO_4 to water; heat and stir until dissolved; add tryptamine, K_2HPO_4 , KH_2PO_4 , yeast extract and agar; adjust pH to 5.7 and autoclave at 121°C for 10 min; add millipore filter sterilized 1:24 aqueous solution of chlorothalanil (Bravo) or 0.2 g/1000 ml of cyclohexamide.

2.2. *Pseudomonas cepacia* cultures.

2.2.1. Collection and storage of *Ps. cepacia* strains.

Cultures of *Ps. cepacia* strains 56, 406, 526 (Table 2.1) were provided by Dr. P.J. Dart, Department of Agriculture, University of Queensland (formerly of Research School of Biological Sciences, ANU). These cultures were stored at 4°C and subcultured every two months on nutrient agar.

2.2.2. Production of *Ps. cepacia* suspension.

In each 250 ml conical flask, 150 ml nutrient broth was inoculated with a strain of *Ps. cepacia* by an inoculation loop from an agar culture. The flask was then incubated in a shaking water bath of 30°C overnight (16 h) to produce bacterial suspension which contained approximately 10^9 colony forming units per milliliter (cfu/ml).

2.3. Cultures of *Pythium* spp. and *Phytophthora* spp.

2.3.1. Collection and storage of fungal cultures.

Several fungal cultures were obtained, including *Phytophthora cinnamomi* A210 from the culture collection of the Division of Forestry and Forest Products, CSIRO; *Ph. cinnamomi* 397, and *Ph. drechsleri* P₁₁ from the Research School of Biological Science, ANU; *Pythium ultimum* P₄₁₆ from the culture collection of the Department of Forestry, ANU (Table 2.1). All these cultures were stored at 4°C and subcultured every 6 months on corn meal agar slopes.

2.3.2. Isolation of *Pythium* spp. and *Phytophthora* spp.

2.3.2.1. Isolation of *Pythium* spp. and *Phytophthora* spp. from soils using cress as bait.

Soils (loamy and sandy) were collected from gardens and also from seedling-beds where seedlings of *Eucalyptus* spp. had been growing each year, dispensed into large petri plates (20 cm diameter). Seeds of cress which were very susceptible to the damping-off caused by *Pythium* spp and *Phytophthora* spp were sown into the petri plates. The petri plates were then watered and covered in order to keep a high humidity, and placed at room temperature. Four days later, the dead or dying plants were collected.

Plants were washed carefully and dried with filter paper. They were surfaced sterilized in 75% alcohol for 30 sec, then rinsed five times with sterile distilled water. The plants were cut into pieces and placed into petri plates that contained 20 ml P₁₀VP selective medium. The petri plates were incubated at 26°C. Fungi that grew out from the plants were transferred onto other P₁₀VP petri plates and subcultured.

2.3.2.2. Isolation of *Pythium* spp. and *Phytophthora* spp. from soils by dilution plate method.

Both loamy and sandy soils were diluted with sterile distilled water by 1:10, 1:100, 1:1000, and 1:10000. The resulting soil suspensions were then plated out on P₁₀VP selective medium. After the petri plates were incubated at 26°C for 2 days, isolates were obtained from 1:10 and 1:100 diluted suspensions of loamy soil. No isolate was obtained from other suspensions.

2.3.2.3. Purification and identification of fungal isolates.

All the fungal isolates were purified by cutting and subculturing their hyphal tips onto P₁₀VP selective medium. Finally, two isolates were identified as *Pythium ultimum* and *Pythium debaryanum* by Professor D. M. Griffin and

numbered as *Pythium ultimum* 861 and *Pythium debaryanum* 862. They were stored at 4°C and subcultured every 6 months on corn meal agar slopes.

2.4. Soil.

2.4.1. Collection of soil.

Soil was collected from an over-mature spotted gum (*Eucalyptus maculata* Hook) forest within the Kioloa State Forest, New South Wales. The surface grass and leaves were removed and soil from the top 30 cm was collected. This soil was sieved through 3 mm mesh and stored at room temperature in plastic bins.

2.4.2. Mechanical analysis of the soil.

This analysis was done according to Corbett (1969). Soil (100 g) was boiled in hydrogen peroxide to remove the organic matter. It was then dried at 105°C for 48 h. Dried soil (60 g) was placed into an electric mixing beaker. 50 ml of 5% calgon, brought to pH 8.5 with sodium carbonate, was added to the beaker. The beaker was two thirds filled with distilled water. The contents were agitated with a mixer (Hamilton Beach) for 10 min. The contents of the beaker were then washed into a measuring cylinder and the cylinder was filled to 1000 ml mark with distilled water. A rubber stopper was placed over the end of the cylinder and the contents of the cylinder were shaken vigorously for 1 min and then allowed to settle. When the shaking ceased, the time was noted.

After 5 min, the weight of soil colloid in suspension was measured using a soil hydrometer. The temperature of the suspension was also recorded.

After 2 h, the hydrometer and temperature readings of the suspension were repeated. The supernatant clay suspension was poured off from the cylinder. The settled sand and silt were washed into a 250 ml beaker. The beaker was filled to about 10 cm with water, stirred and the sand was allowed to settle for 5 min. The supernatant silt suspension was decanted. This was repeated until

the supernatant liquid was clear. The washed sand was dried at 105°C for 48 h and weighed.

It was found that the soil was a sandy loam containing 14.1% clay, 9.1% silt and 76.8% sand.

2.4.3. Control of soil matric potential.

Throughout this study, soil matric potentials were controlled by using 7 cm diameter Buchner funnels with sintered glass plates of fine porosity (porosity 4) as tension plates. The height of the water column between the porous plate and the surface of a water reservoir was adjusted to give the desired matric potential *the middle of the soil column on* (Fig. 2.1). Soil (50 g), either infested or non-infested with pathogen, was placed onto the tension plates. The soil was wetted and allowed to equilibrate for 24 h before plant seeds were sown.

This tensiometer system is accurate in controlling soil matric potentials and is easy to establish. However, the range of soil matric potentials obtained from 0 to -15 kPa in this thesis is relatively narrow compared with those existing in the field or obtainable by use of other more complex control systems. It might be argued that other control system should have been adopted so as to more nearly equate with the full range of field water potentials. To have done so, however, would have further complicated already complex experiments with many interacting variables. Moreover, it was anticipated from the previous literature that the matric potential differences within even the small range used might well have significant effects on *Ps. cepacia*, on the fungal sporangial production and zoospore release and even on the fungal growth. Further, the aim of these experiments was to open up a hitherto little investigated topic and to establish the types of interactions which exist. A wide range of soil water potentials should be considered eventually, but without the time constraints of a Ph.D project.

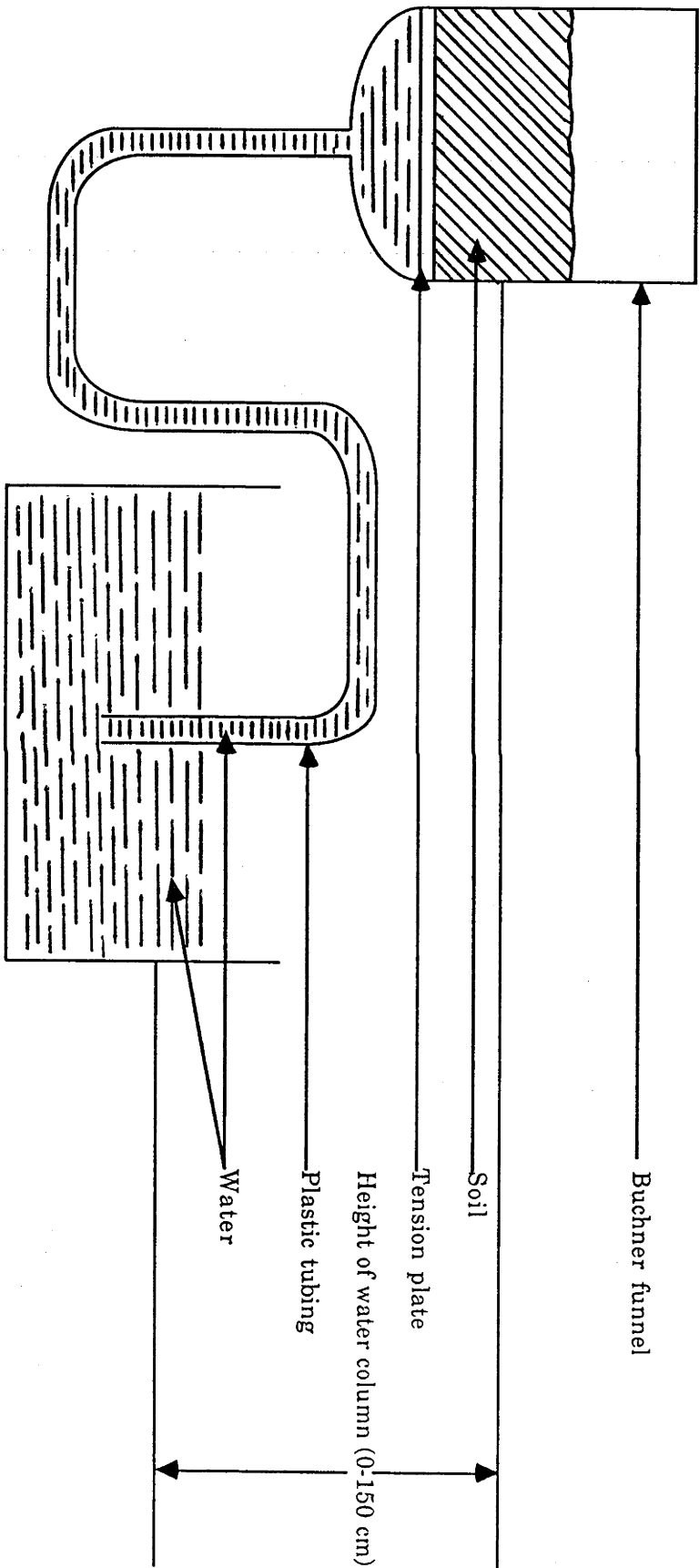
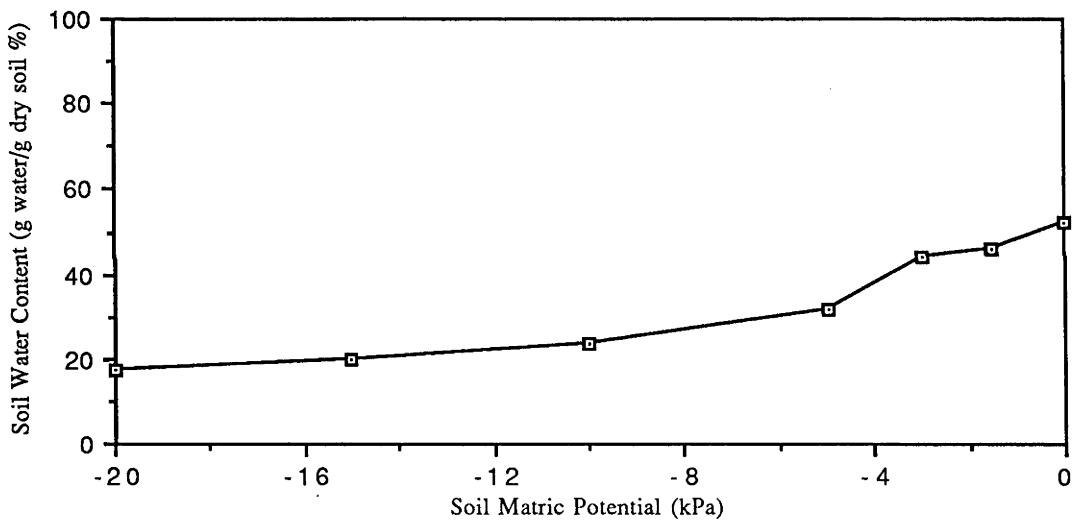


Fig. 2.1. Diagram illustrating apparatus for controlling soil matric potentials.

2.4.4. Soil moisture release curve.

Soil was sampled from each Buchner funnel at various matric potentials and placed into an aluminium cup. The cup and soil were weighed and then oven-dried at 105°C for 48 h. The weight was recorded again. The loss in weight represented the initial moisture content. The soil water content was calculated by dividing the weight of water by the weight of oven-dried soil. The soil moisture release curve (desorption boundary isotherm) is shown by Fig. 2.2.

Fig. 2.2. Soil moisture release curve.



2.5. Host plants.

Seeds of *Pinus radiata* and *Eucalyptus sieberi* were provided by the seed collection, Division of Forestry and Forest Products, CSIRO. Seeds of cress, alfalfa, radish and turnip were bought from ~~the~~ ^a grocery ^{store} (Table 2.1).

Table 2.1. Details of sources of bacteria, fungi and plants

	Origins	Sources
<i>Pseudomonas cepacia</i> 56	Silt loam;	Dr. Peter Dart
	Kempsey, Fredricktown,	Dept. of Agriculture
	NSW	Univ. of Queensland
<i>Pseudomonas cepacia</i> 406	Silt loam	-do-
	Prescott, Madison, USA	
<i>Pseudomonas cepacia</i> 526	-do-	-do-
<i>Phytophthora cinnamomi</i> A210	A ₂ type,	Dr. Ken Old
	<i>Dryandra sessilis</i>	Division of Forestry and Forest Products
	Jarrah forest, Byford, W.A.	CSIRO
<i>Phytophthora cinnamomi</i> 397	unknown	Research School of Biological Science,
		ANU
<i>Phytophthora drechsleri</i> P ₁₁	unknown	-do-
<i>Pythium ultimum</i> P ₄₁₆	unknown	Dept. of Forestry, ANU
<i>Pythium ultimum</i> 861	Loamy soil, ACT	-do-
<i>Pythium debaryanum</i> 862	Loamy soil, ACT	do-
<i>Pinus radiata</i>	unknown	CSIRO seedlot No 13441
<i>Eucalyptus sieberi</i>	unknown	CSIRO seedlot No 12127
<i>Lepidium sativum</i> (cress)	Arthur Yates & Co. PTY. LTD	retail grocery store
<i>Medicago sativa</i> (alfalfa)	-do-	-do-
<i>Raphanus sativus</i> (radish)	-do-	-do-
<i>Brassica rapa</i> (turnip)	-do-	-do-

2.6. Statistical analysis.

Unless otherwise stated, the Genstat programme for analysis of variance (ANOVA, Rothamsted Experimental Station) and Duncan's multiple-range test were used for data analysis. Significance for treatment effects were tested at probability levels of 5, and 1%.

2.7. Preliminary experiments---Development of an experimental system.

Prior to commencing the study of interaction between plant, *Pseudomonas cepacia*, *Pythium* spp. and *Phytophthora* spp., it was essential that an experimental system be established where the host plant, soil and the method of applying the fungal pathogen were all suitable. A series of experiments were thus conducted to develop an acceptable system. Only those experiments of direct relevance to later work are reported here.

2.7.1. The antagonisms of various strains of *Pseudomonas cepacia* against different fungal pathogens.

The antagonistic effects of three *Ps. cepacia* strains (406, 56, 526) were tested against fungal isolates *Phytophthora cinnamomi* A210, *Ph. cinnamomi* 397, *Ph. drechsleri* P₁₁, *Pythium ultimum* P₄₁₆, *Py. ultimum* 861 and *Py. debaryanum* 862. Potato dextrose agar plates were used for the test.

A 2 day old *Ps. cepacia* culture was transferred by a loop onto a 9 cm petri plate with four spots placed evenly round the petri plate, each 5 mm from the edge. The petri plate contained 20 ml potato dextrose agar medium. At the same time, one fungal plug was transferred from a 7 day old culture onto the center of the petri plate using a 5 mm diameter cork borer and forceps. Control plates were inoculated with fungal plugs only. The petri plates were incubated at 26°C for 1 week. The distance from the edge of the *Ps. cepacia* 526 colony to the edge of the fungal colony was measured as the size of the fungal growth inhibition zone.

Fig. 2.3. Fungal growth inhibition caused by *Ps. cepacia* 56 on PDA agar plate.

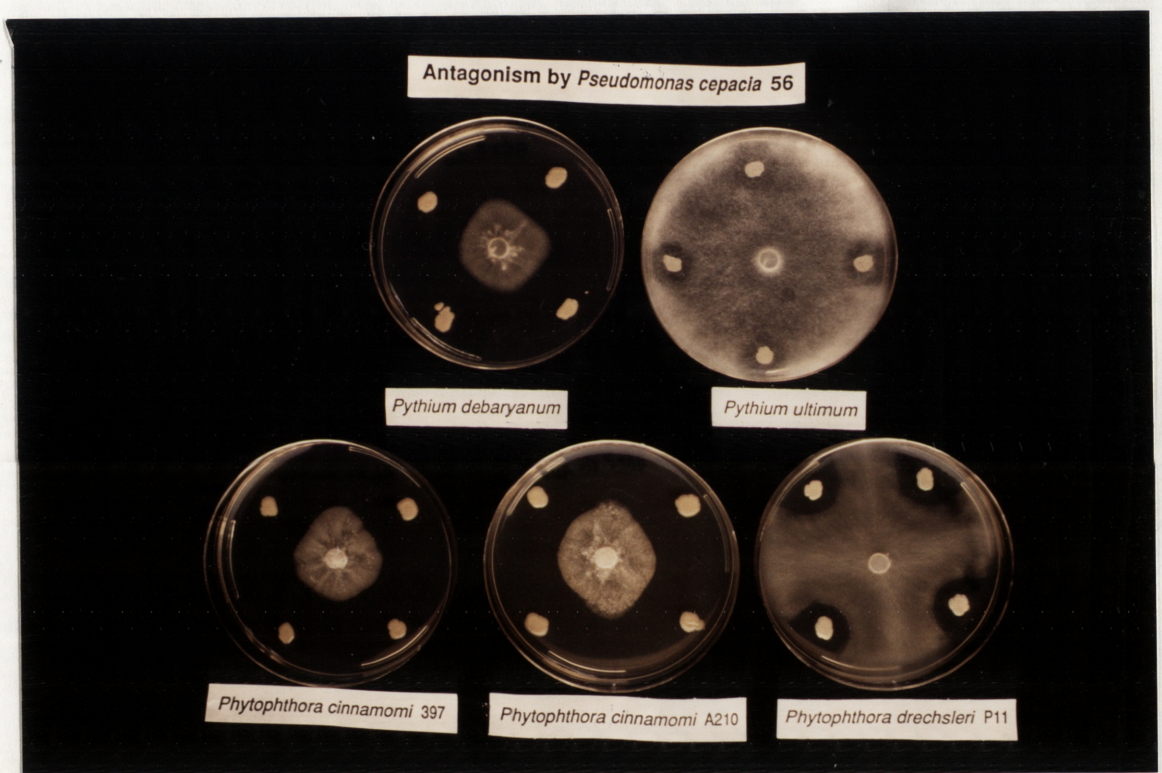


Fig. 2.4. Fungal growth inhibition caused by *Ps. cepacia* 406 on PDA agar plate.

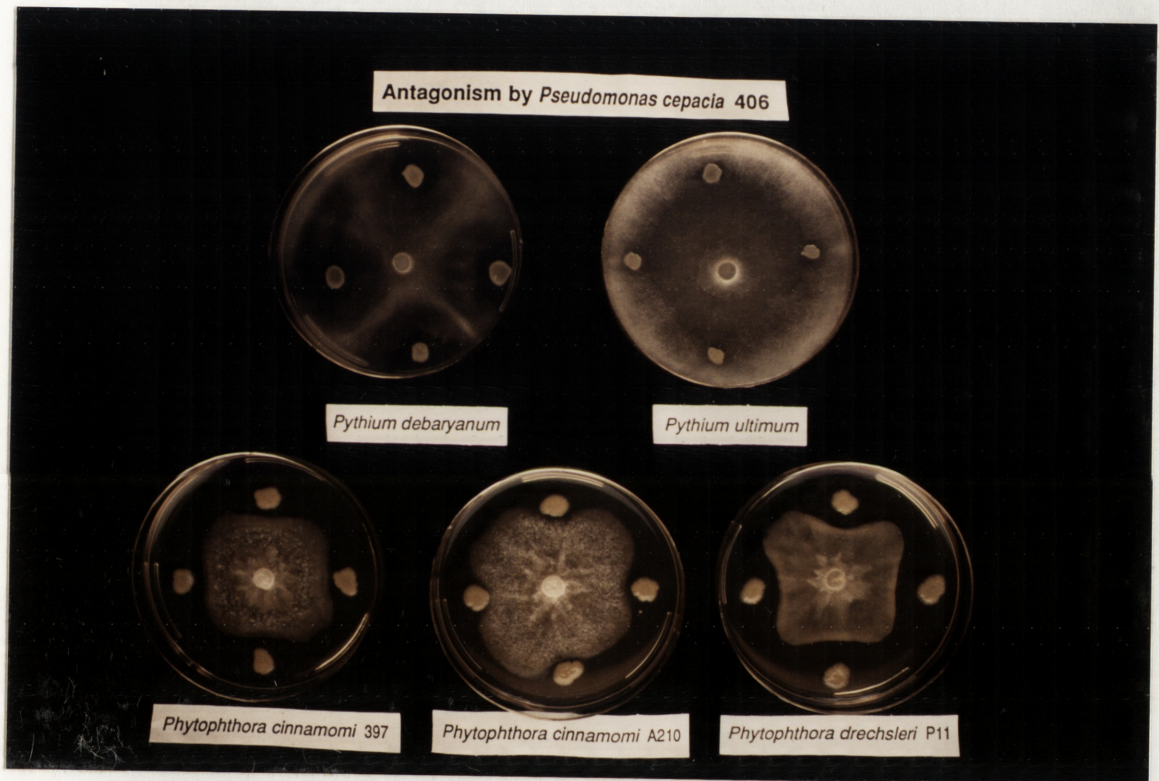
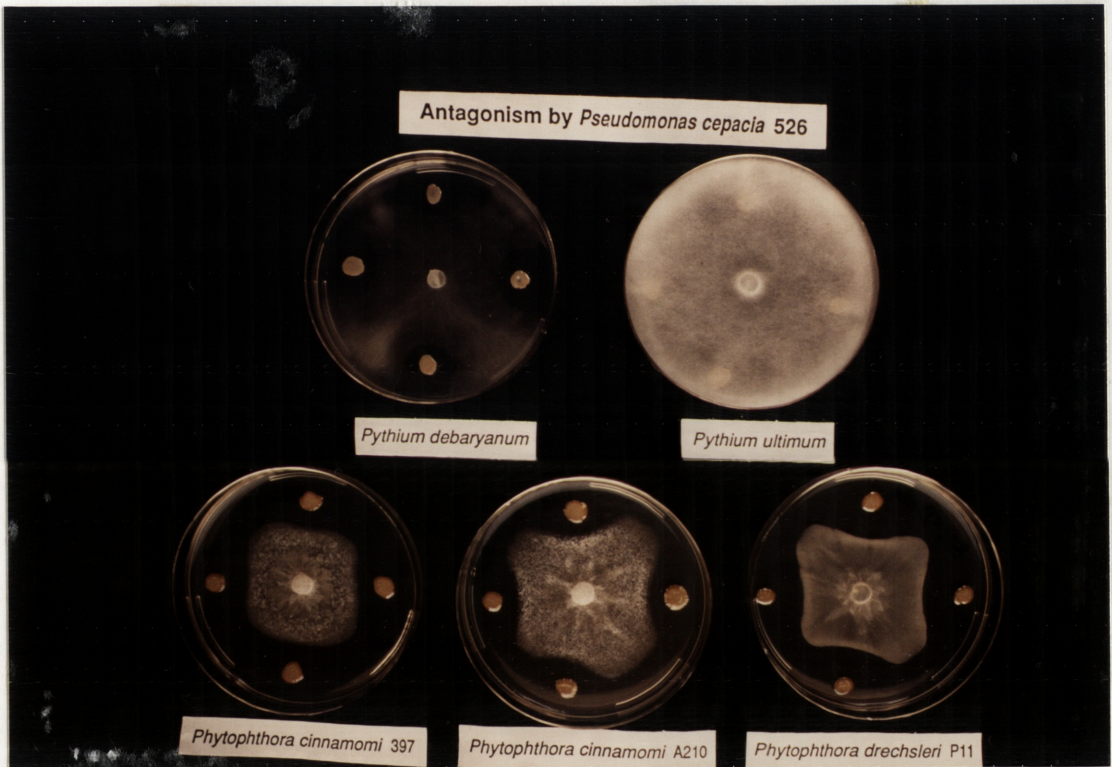


Fig. 2.5. Fungal growth inhibition caused by *Ps. cepacia* 526 on PDA agar plate.



There were three replicate plates for each *Ps. cepacia* strain and fungus combination.

The results showed that the three strains of *Pseudomonas cepacia* had similar patterns of antagonistic effects against the fungal isolates. They all produced growth inhibition zones against *Ph. cinnamomi* A210, *Ph. cinnamomi* 397, *Ph. drechsleri* P₁₁, and *Py. debaryanum* 862. No strain produced a growth inhibition zone against the two *Py. ultimum* isolates (Figures 2.3, 2.4 & 2.5).

2.7.2. Pathogenicity trials for fungal isolates and comparison of several media for fungal inoculum production.

2.7.2.1. Soil and plant.

Soil was sterilized at 121°C, 30 min each day for two consecutive days, mixed and dispensed into pots. The soil was saturated with non-sterile tap water 2 days before plant seeds were sown into the pots. *Pinus radiata* and *Eucalyptus sieberi* were used in separate experiments, with 20 seeds of either being placed into each pot before soil-fungal inoculum mixture were applied to mulch them.

2.7.2.2. Preparation and application of fungal inocula.

Three media for inoculum production of *Phytophthora* spp. and *Pythium* spp. were reported previously and were used in this experiment, including vermiculite V-8 (Chilvers 1962), sand cornmeal (Bertus 1968, Chang & Kommedahl 1968, Lumsden *et al.* 1983), and oat seeds (Dutky & Lumsden 1986, Lumsden *et al.* 1976, Lumsden *et al.* 1979, Lumsden *et al.* 1983, Papavizas *et al.* 1981). To make vermiculite V-8 medium, 50 g washed clean vermiculite were mixed with 50 ml cleared V-8 juice in a 500 ml conical flask. To make sand cornmeal medium, washed and then air dried sand was mixed with cornmeal and water at a ratio of 97 g : 3 g : 15 ml. The sand-cornmeal mixture was then placed into a 500 ml conical flasks. To make oat seed medium, 100 g ^{untreated} oat seeds were put into a 500 ml conical flask and 150 ml distilled water was added. All

media were autoclaved at 121°C for 1 h. Each conical flask containing various media was then inoculated with 3 agar plugs cut from a 7 day old culture of various fungi grown on potato dextrose agar, using a 5 mm diameter cork borer and forceps. The flasks were then incubated at 26°C for 1 month and hand-shaken daily. Inocula were prepared for *Ph. cinnamomi* A210, *Ph. cinnamomi* 397, *Ph. drechsleri* P₁₁, *Py. ultimum* P₄₁₆, *Py. ultimum* 861 and *P. debaryanum* 862.

The vermiculite V-8 in which either *Phytophthora* sp. or *Pythium* sp. had been grown were mixed with sterile soil at 1:100 (w:w) on a soil dry weight basis, after being flooded with sterile distilled water, shaken and decanted six times to remove excess nutrients. Sand cornmeal inoculum and oat seed inoculum were also mixed uniformly with sterile soil at 1:100 (w:w) on a soil dry weight basis. These soil-inoculum mixtures were used to mulch the top soil in pots after plant seeds were placed. Control experiments were conducted by using vermiculite V-8, sand cornmeal or oat seeds without any fungus and by using only soil without vermiculite V-8, sand cornmeal or oat seeds. Four replicates were made of each fungus-medium combination. The pots were watered twice a day at approximately 9:00 am and 4:00 pm. The glasshouse temperature varied daily between 18 and 36°C.

2.7.2.3. Results.

Investigations were made of the seedling emergence rate and the seedling mortality rate 1 month after seedling emergence. The data were analyzed statistically (refer to 2.6).

The results showed that the pathogenicity of the fungi were very much dependent on the way their inocula were produced. Inoculum produced with oat seed medium consistently caused more severe seedling damping-off than did inoculum produced with either vermiculite V-8 or sand cornmeal. When

vermiculite V-8 was used, the color of the soil water draining from the pots became dark brown, possibly due to the release of materials from the vermiculite which had also been found to be a disadvantage before (Chilvers 1962), and plant seedlings were smaller and stunted. When sand cornmeal was used, the fungi failed to produce sufficient propagules in the flasks to cause disease. With all the fungal isolates tested, *Ph. cinnamomi* A210, *Ph. cinnamomi* 397 and *Py. ultimum* 861 were proven to be pathogenic to the plants when their inocula were produced with oat seed. With this inoculum, there was less variable mortality (10-20% above that which occurred in controls). *Ph. cinnamomi* A210 (from hereafter mentioned as *Phytophthora cinnamomi* or *Ph. cinnamomi* in this thesis) and *Pythium ultimum* 861 (from hereafter mentioned as *Pythium ultimum* or *Py. ultimum* in this thesis) were used for further experiments. *Ph. cinnamomi* 397 was not used further because it was not different from *Ph. cinnamomi* A210 in terms of its pathogenicity. Oat seed was chosen as the medium for the production of fungal inocula in later experiments.

2.7.3. Choice of turnip as host plant.

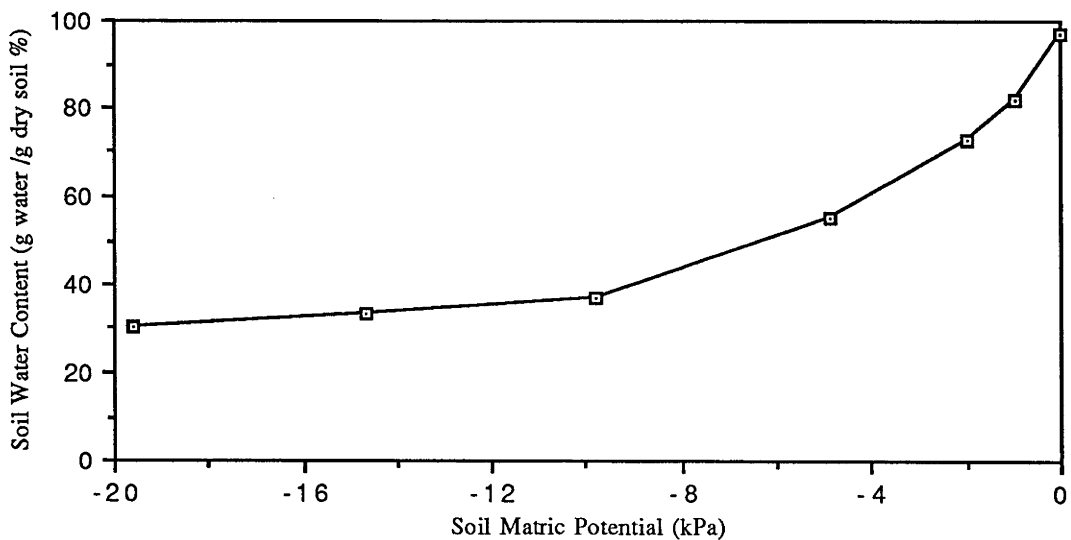
Although both *Pinus radiata* and *Eucalyptus sieberi* showed susceptibility to some of the fungi, it was found that they were too resistant (only 10-20% mortalities) and gave wide variation in results. Experiments with them also lasted too long so that the actual effects of the pathogens may have been masked by the changing soil conditions. The results were hardly reproducible. They were found not to be a satisfactory host plant for the purpose of studying the biological control effects of *Ps. cepacia*. Experiments were thus done with cress, alfalfa, radish and turnip attempting to find a more suitable host plant. Oat seed inocula of *Phytophthora cinnamomi* and *Pythium ultimum* were tested on these plants using the same method as described in 2.7.2. Consequently, *Ph. cinnamomi* caused mortalities of 88% cress, 53% turnip, 23% alfalfa and 17% radish,

whereas *Py. ultimum* caused mortalities of 98% cress, 73% turnip, 33% alfalfa and 35% radish, relative to controls. Turnip was therefore chosen as a susceptible host plant suitable for further studies with both pathogens.

2.7.4. Treatment of soil.

It was found that the soil was not suitable for the experimental system. The surface soil dried rapidly when the glasshouse temperature was high, resulting in highly variable soil conditions within each pot in one day. Disease incidences were variable. The plant seed germination rate was low and plant seedlings grew poorly. A further treatment was therefore used (Bateman 1961, Brown & Kennedy 1966, Broadbent *et al.* 1977, Dunleavy 1955, Matheron & Mircetich 1985, Matheron & Mircetich 1985) by mixing the soil with milled peat moss (Warrior, Southland Peat Limited, Invercargill, New Zealand) at 1:1 (V:V).

Fig. 2.6. Moisture release curve of the soil-peat mixture.



This soil-peat mixture proved to be a suitable medium. The addition of the peat increased the soil water holding ability and the soil gas exchange ability. Plants were not harmed. This soil-peat mixture has been used throughout this study. *but did not change the pH*

From hereafter, unless otherwise mentioned, the 'soil' in this thesis refer to the soil-peat mixture. The moisture release curve of this soil-peat mixture is shown

in ~~by~~ Fig. 2.6.

**CHAPTER 3. THE PATHOGENICITIES OF *PHYTOPHTHORA CINNAMOMI*
AND *PYTHIUM ULTIMUM* AND THE EFFECTS OF *PSEUDOMONAS CEPACIA* 526
AT VARIOUS SOIL TEMPERATURES AND SOIL MATRIC POTENTIALS**

3.1. Introduction.

Phytophthora cinnamomi and *Pythium ultimum* are important plant pathogens (refer to 1.1). Attempts to apply biological control to diseases caused by *Ph. cinnamomi* and *Py. ultimum* have been made, but no commercial product is available (refer to 1.3). *Pseudomonas cepacia* has been reported as a potential biological control bacterium (refer to 1.3.2). No research, however, has been conducted on the biological control by *Ps. cepacia* of diseases caused by *Ph. cinnamomi* (refer to 1.3.2). In the preliminary experiments in chapter 2, the antagonistic effects of *Ps. cepacia* 526 against *Ph. cinnamomi* and *Py. ultimum* have been studied in culture. In this chapter, experiments were conducted to study the pathogenicities of the fungi and the plant growth-promoting and disease biological control effects of *Ps. cepacia* 526 in soil and their associations with soil temperature and soil matric potentials.

3.2. Materials and Methods.

3.2.1. *Pseudomonas cepacia* 526 nutrient broth suspensions of approximately 10^9 cfu/ml were prepared as described in 2.2.2. One month old oat seed inocula of both *Phytophthora cinnamomi* and *Pythium ultimum* were prepared as described in 2.7.2.2. Turnip was used as host plant (refer to table 2.1 and 2.7.3). The non-sterile soil-peat mixture was used as plant growth medium (refer to 2.7.4).

3.2.2. A series of experiments were initially conducted in a glasshouse where the air temperatures ranged daily from 17.5°C to 36°C, the light was normal

The photoperiod was not recorded.

sunlight. Since results which are reported in later sections showed that the high temperature in the glasshouse had played major roles in the soil temperature changes which in turn affected the disease incidences, a second series of experiments were conducted at lower temperature in a constant temperature room. In the constant temperature room, the daily air temperature ranged from 24°C to 26°C, the light was artificial fluorescent light (40 w at each matric potential level, Fig. 3.1). *The photoperiod was controlled by a timer at 12h:12h.*

Soil matric potential was controlled by using 7 cm diameter Buchner funnels with sintered glass plates of fine porosity (porosity 4) as tension plates. The Buchner funnels were connected to water reservoirs by plastic tubings. The height of the water column between the porous plate and the surface of a water reservoir was adjusted to give the desired matric potential (Fig. 2.1, Fig. 3.1.).

Oat seed inoculum of either *Ph. cinnamomi* or *Py. ultimum* grown in conical flasks was picked out and put into the soil at selected rates 1 day before the soil was placed into the Buchner funnels. Since only a few kilograms of soil was needed for one experiment, the soil and the oat seed inoculum could be mixed uniformly in a plastic bag by hand-rubbing the mixture from outside the bag while the oat seed aggregates were broken up. This soil infestation method was proven to be effective by other workers (Dutky & Lumsden 1986, Lumsden *et al.* 1976, Lumsden *et al.* 1979, Lumsden *et al.* 1983, Papavizas *et al.* 1981) and also has given consistent results in my series of experiments.

Soil (50 g), with or without the pathogen, was then placed in a Buchner funnel, wetted and allowed to equilibrate for 24 h. Turnip seeds (30) were sown into the Buchner funnel and covered with a thin layer of soil. *Ps. cepacia* 526 nutrient broth suspension (5 ml) was then sprayed into each Buchner funnel. More water was sprayed into each Buchner funnel to saturate the soil so that the bacterium ~~was~~ drained into the soil uniformly during the quick process of water

equilibration
~~equilibrium~~

Nutrient broth (5 ml) without *Ps. cepacia* 526 was sprayed into each control Buchner funnel. In some experiments, the Buchner funnels were covered with plastic food wrap to prevent heterogeneity of water distribution through evaporation. There were three replicates for each fungus-bacterium-soil matric potential treatment.

During the experiments, water samples were taken from the water reservoirs and tested for the presence of either *Ph. cinnamomi* or *Py. ultimum* or *Ps. cepacia* 526. The results was negative, indicating that none of them can pass through the tension plates.

3.2.2.1. Experiments in the glasshouse.

Two experiments were conducted in a glasshouse, one with *Ph. cinnamomi* and the other with *Py. ultimum*. Fungal inocula were added to the soil at 0, 1.0 and 1.5%. Soil matric potentials were controlled at 0, -1.5, -3.0, -5.0, -10.0 and -15.0 kPa. *Ps. cepacia* 526 was applied as described above.

3.2.2.2. Experiments in the constant temperature room.

In all experiments conducted in the constant temperature room, soil matric potentials were controlled at 0, -1.5, -3.0, -5.0 and -10.0 kPa.

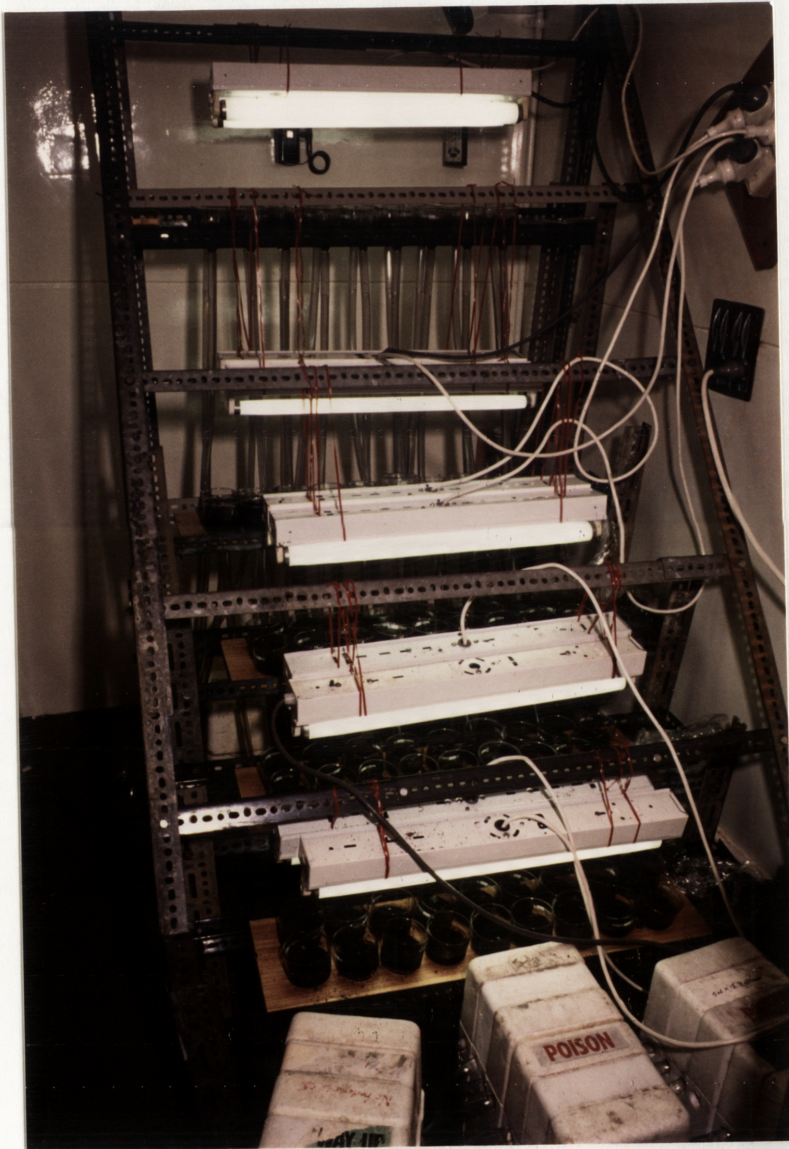
Experiment 1: Inoculum of *Ph. cinnamomi* was added to the soil at 0, 1.0 and 1.5%. *Ps. cepacia* 526 was applied as described above in this chapter.

Experiment 2: This is a ~~replicate~~
~~repeat~~ of experiment 1, except that the Buchner funnels were covered with plastic food wrap to prevent heterogeneity of water distribution through evaporation.

Experiment 3: Inoculum of *Ph. cinnamomi* was added to the soil at 0, 0.2, 0.4, 0.6, 0.8 and 1.0% without *Ps. cepacia* 526.

Experiment 4: Inoculum of *Py. ultimum* was added to the soil at 0, 0.5 and 1.0%. *Ps. cepacia* 526 was applied as described above in this chapter.

Fig. 3.1. Apparatus to control soil matric potentials



Experiment 5: Inoculum of *Py. ultimum* was added to the soil at 0, 0.1 and 0.5%. *Ps. cepacia* 526 was also applied. The Buchner funnels were covered with plastic food wrap to prevent heterogeneity of water distribution through evaporation.

Experiment 6: Inoculum of *Py. ultimum* was added to the soil at 0, 0.1, 0.2, 0.3, 0.4 and 0.5%. *Ps. cepacia* 526 was not used.

3.2.3. Measurement of soil water content and soil temperature.

Measurement of soil water content at various matric potentials was done according to the method described in 2.4.4. Soil temperatures at various soil matric potentials were measured ~~consecutively~~ *continuously* using copper-~~constantin~~ *constantan* thermocouple temperature sensors connected to a chart recorder (Leeds & Northrup speedomax).

3.2.4. Investigation of the damping-off of turnip.

The fungi infected turnip seeds and seedlings causing pre- and post-emergence damping-off. When the seed was infected, it failed to emerge. When the seedling was infected, the basal stem became dark brown and shrank and the seedling fell over. The infection happened mostly in the first two weeks. However, in this series of experiments, to record the disease separately as pre- and post-emergence damping-off would be misleading, because the number of post-emergence deaths were very different due to the differences in the initial numbers of seedlings which had emerged rather than to differences in fungal pathogenesis. Therefore, in these experiments, the numbers of plant seedlings surviving two weeks after plant seeds were sown were investigated and used as indicators of overall fungal pathogenicity. The numbers were converted into percentage of seedling survival relative to number of seeds sown. An angular transformation [$\sin^{-1}(\text{square root of seedling survival rate})$] was applied to the data. The data were analyzed by analysis of variance (refer to 2.6).

3.2.5. Measurement of plant growth effects.

At the conclusion of the investigation for the numbers of surviving seedlings, the seedlings together with soil were pushed out from each Buchner funnel by water pressure from the other side of the tension plate. The seedlings from the same Buchner funnel were rinsed and washed carefully in tap water to remove the soil attached to the roots, then cut into two parts (root and shoot), dried to a constant weight at 75°C and the parts weighed. This was done separately for the three replicated funnels.

It was found that the plant root weight was so small in contrast to the plant shoot weight that a major relative change in the plant root weight had little influence on the whole plant weight. In other words, the plant shoot weight was nearly equal to the whole plant weight. Therefore only the plant weight and the plant root weight were used as the measurement of plant growth. A natural logarithm-transformation ($\ln(x)$) was applied to the data. The data were analyzed statistically (refer to 2.6).

3.2.6. Recovery of *Ph. cinnamomi* and *Py. ultimum* from diseased plants.

To confirm that diseases were caused by the fungi applied, the diseased seedlings were collected. These seedlings were washed carefully and dried with filter paper. The shoot was cut off. Surface sterilization of the plant root together with the basal part of the stem was effected by placing it in 75% alcohol for 1 min, then rinsing with sterile distilled water five times. The plant root with the basal stem was cut into pieces and placed onto petri plate that contained 20 ml P₁₀VP selective medium (refer to 2.1.7). The petri plates were incubated at 26°C and checked for the growth of the fungi.

3.3. Results.

3.3.1. Soil temperature and soil water content .

The glasshouse air and soil temperatures are depicted in Fig. 3.2 which reveals a complex situation. Over much of the diurnal period, all the air and soil temperatures were very close. However, the soils at -5.0, -10.0 and -15.0 kPa had higher temperatures than the surrounding air between 2:00 pm and 9:00 pm, whereas soils at 0 and -1.5 kPa between 4:00 pm and 6:00 pm were cooler than the surrounding air. Further, the rate of temperature change over the period was greater in the lower potential soils. The basis of these differences is discussed in 3.4.2 below.

In the constant temperature room, there were virtually no difference in the daily soil temperatures between the various matric potentials. The daily soil temperatures ranged from 23.5 to 25°C at 0 and -1.5 kPa, from 23.5 to 26°C at -3.0, -5.0 and -10.0 kPa (Fig. 3.3).

The soil water contents were not affected by the air temperatures. At the same matric potential, the soil water content in the glasshouse and that in the constant temperature room were the same (Fig. 3.4).

3.3.2. The effects of soil matric potentials on the host plant

In most experiments, soil matric potential had consistent effects on the host plant. The seedling survival rate was significantly lower at 0 kPa than at other potentials (Figures 3.5a, 3.6a, 3.7, 3.8a, 3.9a, 3.10, 3.11a & 3.12). Both plant weight (Figures 3.5b & 3.6b) and plant root weight (Figures 3.5c, 3.6c, 3.8c, 3.9b & 3.11b) became larger as soil matric potential decreased. The exceptional results were obtained only from the experiment in the constant temperature room using *Ph. cinnamomi* at 6 inoculum levels, in which the seedling survival was significantly lower at -1.5 kPa as well as at 0 kPa (Fig. 3.8a) and the plant weight was not affected by soil matric potentials tested (Fig. 3.8b).

Fig. 3.2. Soil temperatures in the glasshouse.

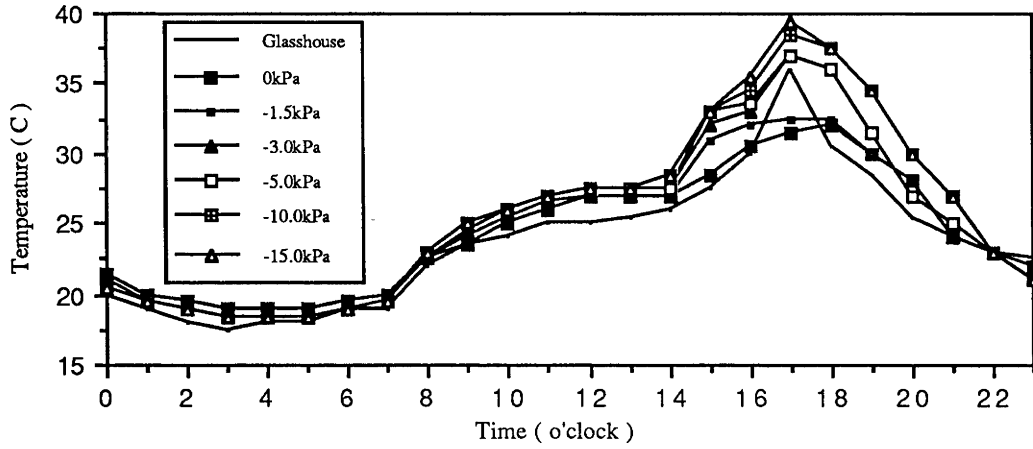


Fig. 3.3. Soil temperatures in the constant temperature room.

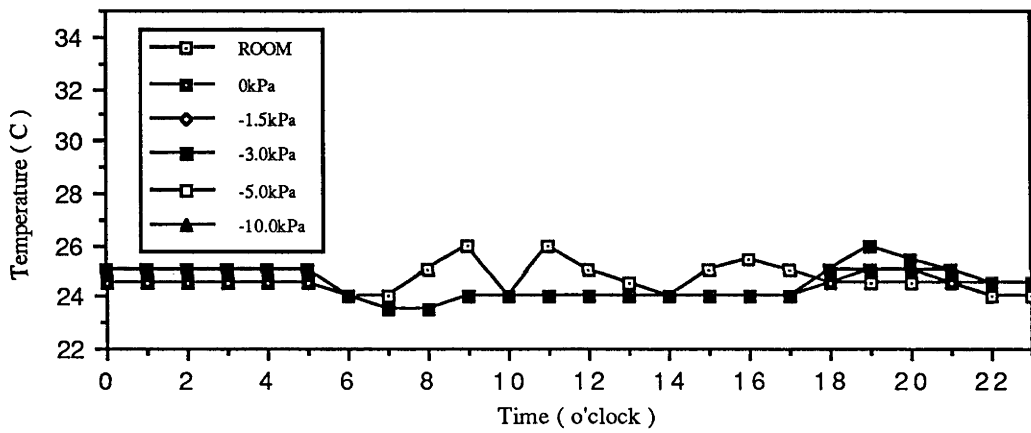


Fig. 3.4. Soil moisture release curve

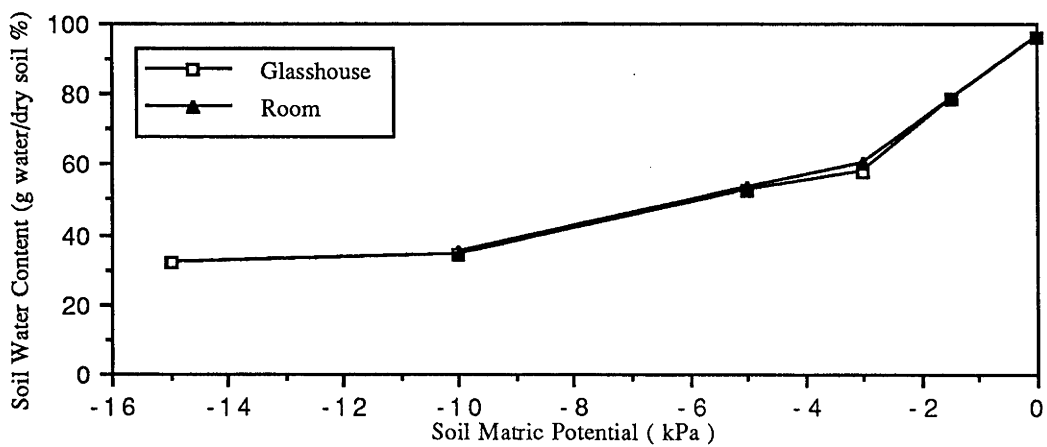
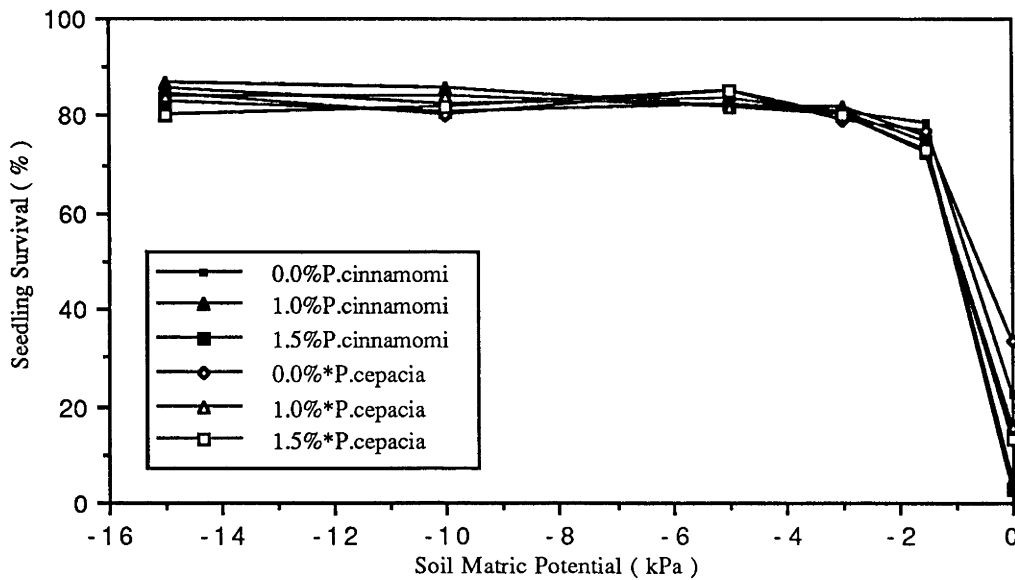


Fig. 3.5a. Effect of *Phytophthora cinnamomi* and *Pseudomonas cepacia* 526 on turnip seedling survival in the glasshouse.



In all figures in this chapter, % only refer to *Ph. cinnamomi*, *P. cepacia* refer to *Ps. cepacia* 526 present at constant inoculum level in the particular experiment.

*****ANALYSIS OF VARIANCE*****

Variate: \sin^{-1} (square root of seedling survival rate)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	5	40230.28	90.92	8040.06	297.78	3.29 (P=0.01)
<i>Ph. cinnamomi</i>	2	385.52	0.87	192.76	7.14	4.92 (P=0.01)
<i>Ps. cepacia</i>	1	63.22	0.14	63.22	2.34	
Potential* <i>Ph. cinnamomi</i>	10	1019.10	2.30	101.91	3.78	2.59 (P=0.01)
Potential* <i>Ps. cepacia</i>	5	440.70	1.00	88.14	3.27	2.35 (P=0.05)
<i>Ph. cinnamomi</i> * <i>Ps. cepacia</i>	2	64.38	0.15	32.19	1.19	

Potential

*

Ph. cinnamomi

*

Ps. cepacia

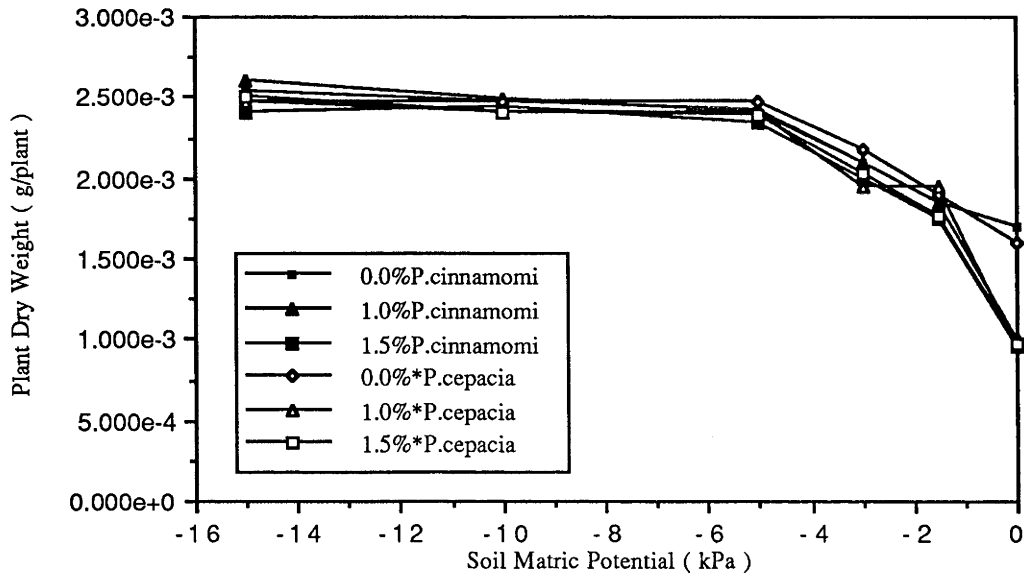
10 102.79 0.23 10.28 0.38

Residual 72 1943.94 4.39 27.00

Total 107 44249.93 100 413.55

SE=5.196, CV%=9.5

Fig. 3.5b. Effect of *Phytophthora cinnamomi* and *Pseudomonas cepacia* 526 on plant weight in the glasshouse.



*****ANALYSIS OF VARIANCE*****

Variate: ln(plant weight)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	5	11.81249	79.40	2.36250	116.782	3.29 (P=0.01)
<i>Ph. cinnamomi</i>	2	0.73366	4.93	0.36683	18.133	4.92 (P=0.01)
<i>Ps. cepacia</i>	1	0.00000	0.00	0.00000	0.000	
Potential* <i>Ph. cinnamomi</i>	10	0.42538	2.86	0.04254	2.103	1.97 (P=0.05)
Potential* <i>Ps. cepacia</i>	5	0.08574	0.58	0.01715	0.848	
<i>Ph. cinnamomi</i> * <i>Ps. cepacia</i>	2	0.06689	0.45	0.03345	1.653	

Potential

*

Ph. cinnamomi

*

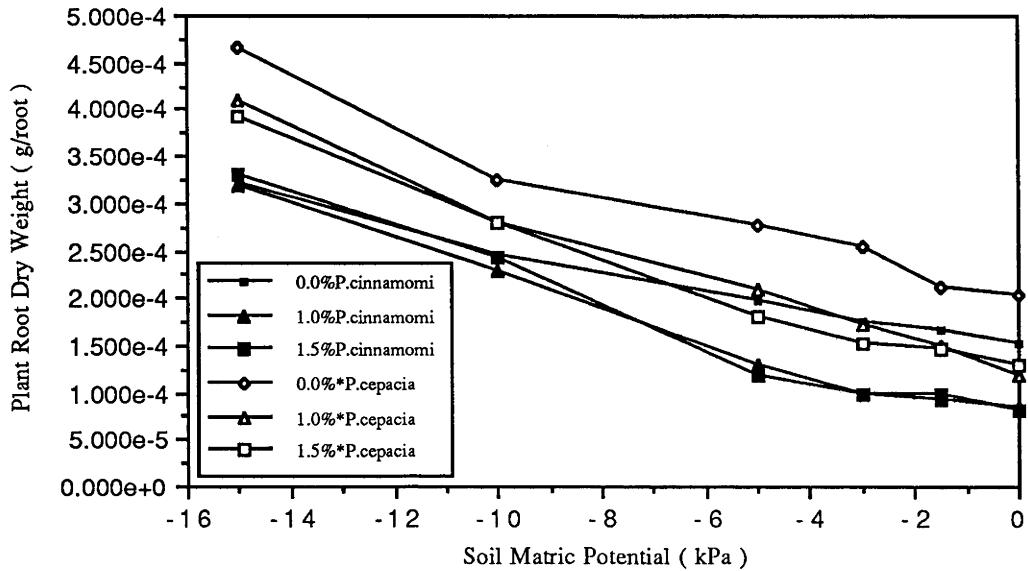
<i>Ps. cepacia</i>	10	0.29530	1.99	0.02953	1.460
--------------------	----	---------	------	---------	-------

Residual	72	1.45656	9.79	0.02023
----------	----	---------	------	---------

Total	107	14.87602	100	0.13903
-------	-----	----------	-----	---------

SE=0.1422, CV%=2.2

Fig. 3.5c. Effect of *Phytophthora cinnamomi* and *Pseudomonas cepacia* 526 on plant root weight in the glasshouse.



*****ANALYSIS OF VARIANCE*****

Variate: ln(plant root weight)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	5	6.21239	44.58	1.24248	55.148	3.29 (P=0.01)
<i>Ph. cinnamomi</i>	2	4.38589	31.47	2.19295	97.335	4.92 (P=0.01)
<i>Ps. cepacia</i>	1	0.65630	4.71	0.65630	29.130	7.01 (P=0.01)
Potential* <i>Ph. cinnamomi</i>	10	0.71117	5.10	0.07112	3.157	2.59 (P=0.01)
Potential* <i>Ps. cepacia</i>	5	0.13643	0.98	0.02729	1.211	
<i>Ph. cinnamomi</i> * <i>Ps. cepacia</i>	2	0.05214	0.37	0.02607	1.157	

Potential

*

Ph. cinnamomi

*

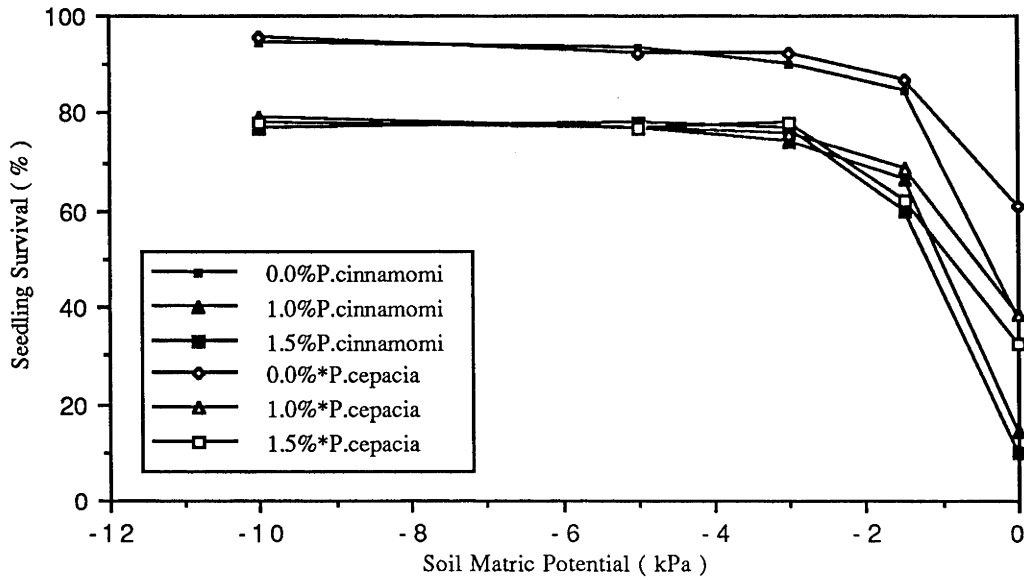
Ps. cepacia 10 0.1605 1.15 0.01605 0.712

Residual 72 1.62216 11.64 0.02253

Total 107 13.93689 100 0.13025

SE=0.1501, CV%=1.7

Fig. 3.6a. Effect of *Phytophthora cinnamomi* and *Pseudomonas cepacia* 526 on turnip seedling survival in the constant temperature room.



*****ANALYSIS OF VARIANCE*****

Variate: \sin^{-1} (square root of seedling survival rate)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	4	12722.83	64.19	3180.71	195.81	3.65 (P=0.01)
<i>Ph. cinnamomi</i>	2	3941.17	19.88	1970.58	121.31	4.98 (P=0.01)
<i>Ps. cepacia</i>	1	775.15	3.91	775.15	47.72	7.08 (P=0.01)
Potential* <i>Ph. cinnamomi</i>	8	179.86	0.91	22.48	1.38	
Potential* <i>Ps. cepacia</i>	4	891.28	4.50	222.82	13.72	3.65 (P=0.01)
<i>Ph. cinnamomi</i> * <i>Ps. cepacia</i>	2	97.34	0.49	48.67	2.996	

Potential

*

Ph. cinnamomi

*

Ps. cepacia

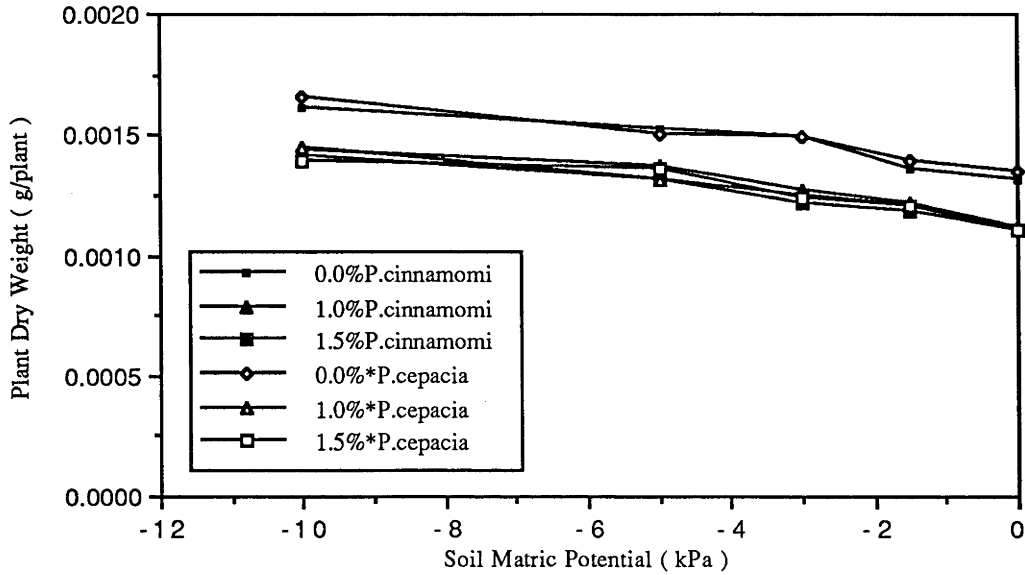
8 239.05 1.21 29.88 1.84

Residual 60 974.62 4.92 16.24

Total 89 19821.30 100 222.71

SE=4.030, CV%=6.9

Fig. 3.6b. Effect of *Phytophthora cinnamomi* and *Pseudomonas cepacia* 526 on plant weight in the constant temperature room.



*****ANALYSIS OF VARIANCE*****

Variate: ln(plant weight)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	4	0.666714	42.39	0.166679	27.19	3.65 (P=0.01)
<i>Ph. cinnamomi</i>	2	0.493600	31.38	0.246800	40.26	4.98 (P=0.01)
<i>Ps. cepacia</i>	1	0.001389	0.09	0.001389	0.23	
Potential* <i>Ph. cinnamomi</i>	8	0.021807	1.39	0.002726	0.44	
Potential* <i>Ps. cepacia</i>	4	0.006361	0.40	0.001590	0.26	
<i>Ph. cinnamomi</i> * <i>Ps. cepacia</i>	2	0.007687	0.49	0.003844	0.63	

Potential

*

Ph. cinnamomi

*

Ps. cepacia

8 0.007433 0.47 0.000929 0.15

Residual

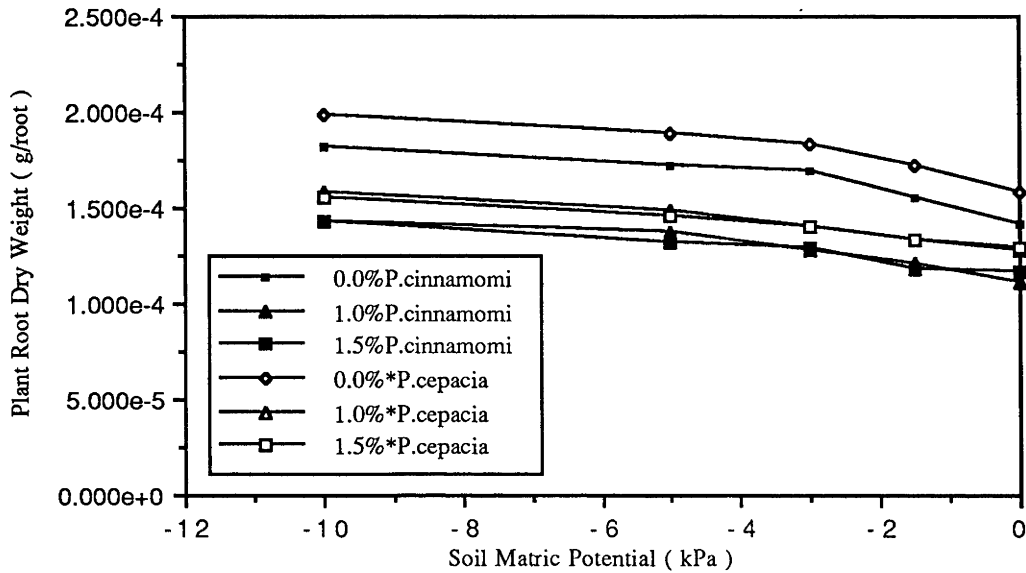
60 0.367839 23.39 0.006131

Total

89 1.572830 100 0.017672

SE=0.07830, CV%=1.2

Fig. 3.6c. Effect of *Phytophthora cinnamomi* and *Pseudomonas cepacia* 526 on plant root weight in the constant temperature room.



*****ANALYSIS OF VARIANCE*****

Variate: ln(plant root weight)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	4	0.655564	43.87	0.163891	34.47	3.65 (P=0.01)
<i>Ph. cinnamomi</i>	2	0.483911	32.38	0.241956	50.90	4.98 (P=0.01)
<i>Ps. cepacia</i>	1	0.025632	1.72	0.025632	5.39	4.00 (P=0.05)
Potential* <i>Ph. cinnamomi</i>	8	0.021714	1.45	0.002714	0.57	
Potential* <i>Ps. cepacia</i>	4	0.007074	0.47	0.001769	0.37	
<i>Ph. cinnamomi</i> * <i>Ps. cepacia</i>	2	0.007880	0.53	0.003940	0.83	

Potential

*

Ph. cinnamomi

*

Ps. cepacia

8 0.007436 0.50 0.000930 0.20

Residual

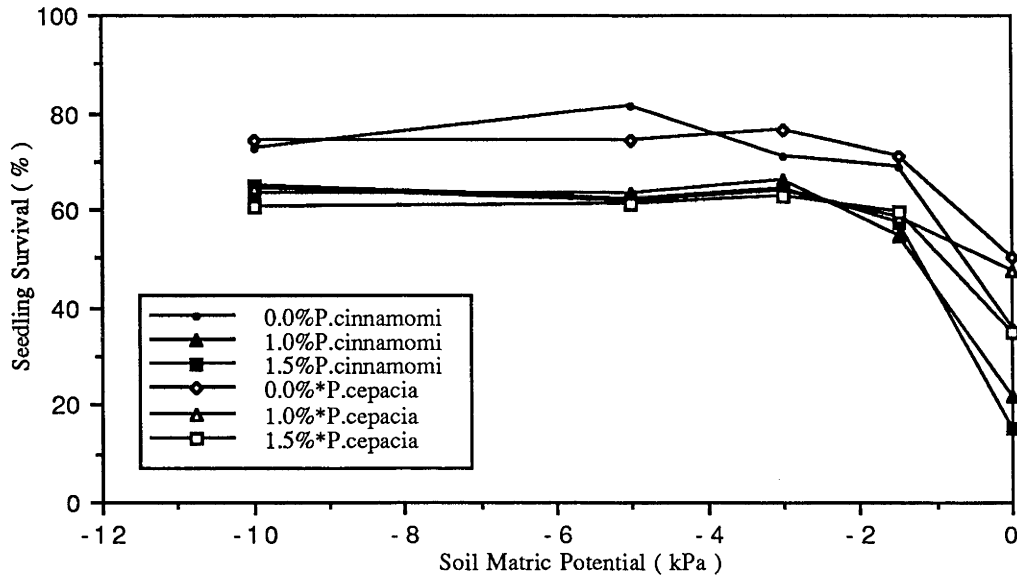
60 0.285227 19.09 0.004754

Total

89 1.494438 100 0.016791

SE=0.06895, CV%=0.8

Fig. 3.7. Effect of *Phytophthora cinnamomi* and *Pseudomonas cepacia* 526 on turnip seedling survival in the constant temperature room.



*****ANALYSIS OF VARIANCE*****

Variate: \sin^{-1} (square root of seedling survival rate)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	4	14378.88	62.78	3594.72	89.01	3.65 (P=0.01)
<i>Ph. cinnamomi</i>	2	3275.39	14.30	1637.70	40.55	4.98 (P=0.01)
<i>Ps. cepacia</i>	1	679.80	2.97	679.80	16.83	7.08 (P=0.01)
Potential* <i>Ph. cinnamomi</i>	8	371.43	1.62	46.43	1.15	
Potential* <i>Ps. cepacia</i>	4	1298.23	5.67	324.56	8.04	3.65 (P=0.01)
<i>Ph. cinnamomi</i> * <i>Ps. cepacia</i>	2	86.90	0.38	43.45	1.07	

Potential

*

Ph. cinnamomi

*

Ps. cepacia

8

388.09

1.69

48.51

1.20

Residual

60

2423.14

10.58

40.39

Total

89

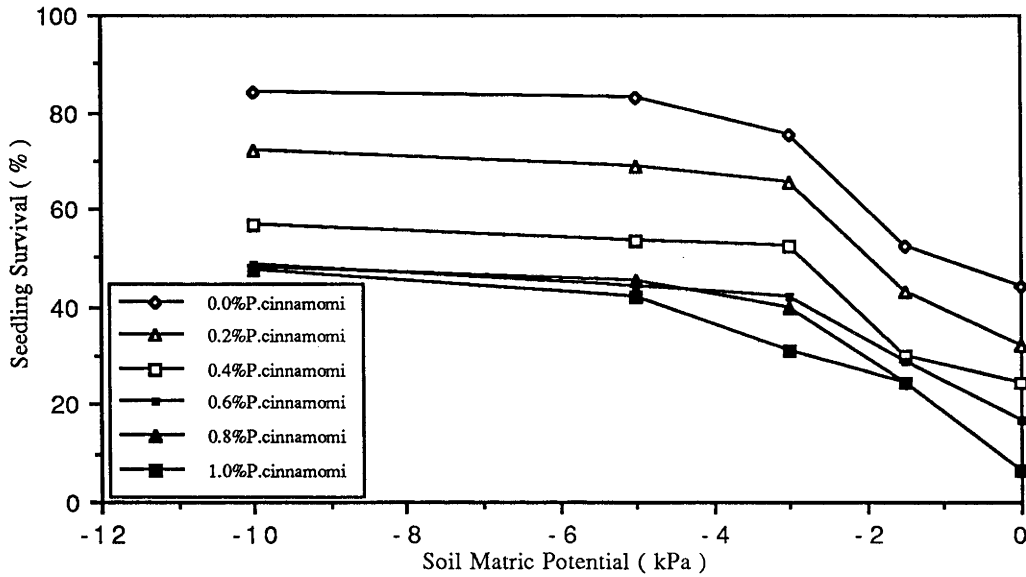
22901.85

100

257.32

SE=6.355, CV%=10.7

Fig. 3.8a. Effect of *Phytophthora cinnamomi* on turnip seedling survival in the constant temperature room.



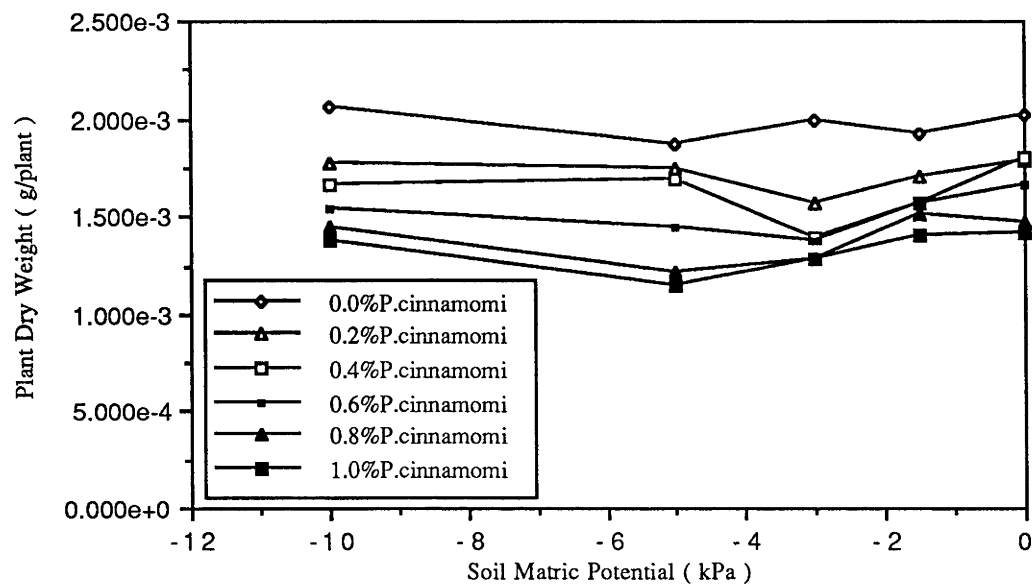
*****ANALYSIS OF VARIANCE*****

Variate: \sin^{-1} (square root of seedling survival rate)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	4	7719.48	41.38	1929.87	33.32	3.65 (P=0.01)
<i>Ph. cinnamomi</i>	5	6326.25	33.91	1265.25	21.85	3.34 (P=0.01)
Potentials* <i>Ph. cinnamomi</i>	20	1134.01	6.08	56.70	0.98	
Residual	60	3474.89	18.63	57.91		
Total	89	18654.63	100.00	209.60		

SE=7.610, CV%=18.7

Fig. 3.8b. Effect of *Phytophthora cinnamomi* on plant weight in the constant temperature room.



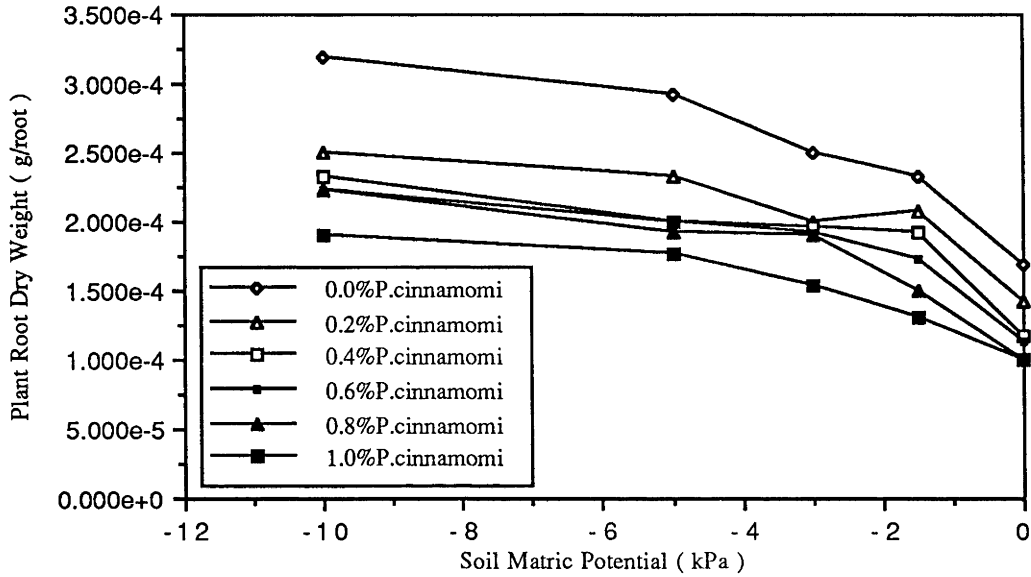
*****ANALYSIS OF VARIANCE*****

Variate: ln(plant weight)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	4	0.25248	6.87	0.06312	2.34	
<i>Ph. cinnamomi</i>	5	1.62035	44.10	0.32407	12.00	3.34 (P=0.01)
Potentials* <i>Ph. cinnamomi</i>	20	0.18446	5.03	0.00922	0.34	
Residual	60	1.61731	44.01	0.02700		
Total	89	3.67461	100	0.04129		

SE=0.1302, CV%=2.0

Fig. 3.8c. Effect of *Phytophthora cinnamomi* on plant root weight in the constant temperature room.



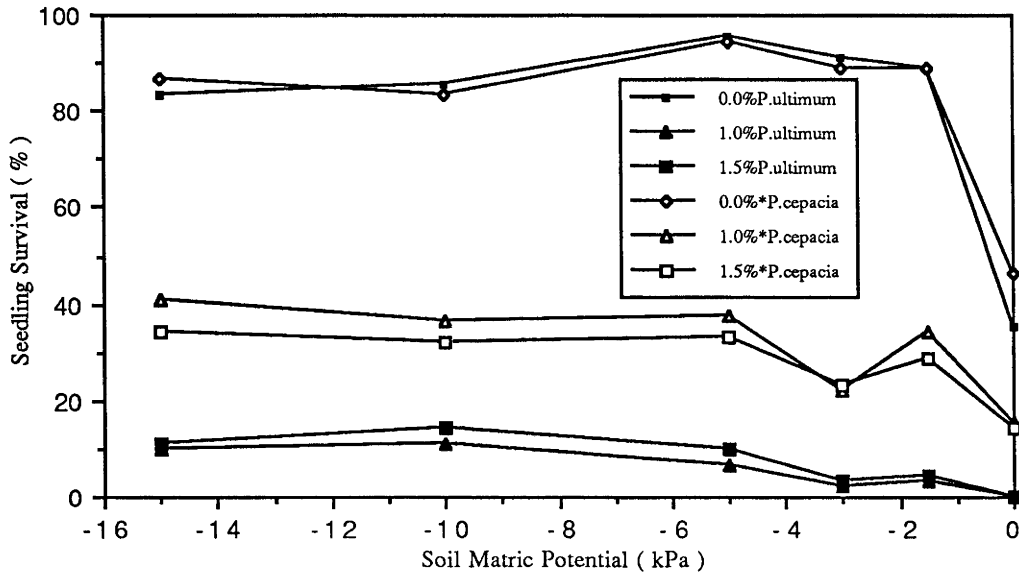
*****ANALYSIS OF VARIANCE*****

Variate: ln(plant root weight)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	4	4.51565	45.66	1.12891	28.20	3.65 (P=0.01)
<i>Ph. cinnamomi</i>	5	2.62058	26.50	0.52412	13.09	3.34 (P=0.01)
Potentials* <i>Ph. cinnamomi</i>	20	0.35144	3.55	0.01757	0.44	
Residual	60	2.40212	24.29	0.04004		
Total	89	9.88981	100	0.11112		

SE=0.2001, CV%=2.3

Fig. 3.9a. Effect of *Pythium ultimum* and *Pseudomonas cepacia* 526 on turnip seedling survival in the glasshouse



*****ANALYSIS OF VARIANCE*****

Variate: \sin^{-1} (square root of seedling survival rate)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	5	5186.91	9.61	1037.38	46.98	3.29 (P=0.01)
<i>Py. ultimum</i>	2	42571.91	78.91	21285.96	964.04	4.92 (P=0.01)
<i>Ps. cepacia</i>	1	2220.03	4.11	2220.03	100.54	7.01 (P=0.01)
Potential* <i>Py. ultimum</i>	10	324.94	0.60	32.49	1.47	
Potential* <i>Ps. cepacia</i>	5	228.00	0.42	45.60	2.07	
<i>Py. ultimum</i> * <i>Ps. cepacia</i>	2	1427.13	2.65	713.57	32.32	4.92 (P=0.01)

Potential

*

Py. ultimum

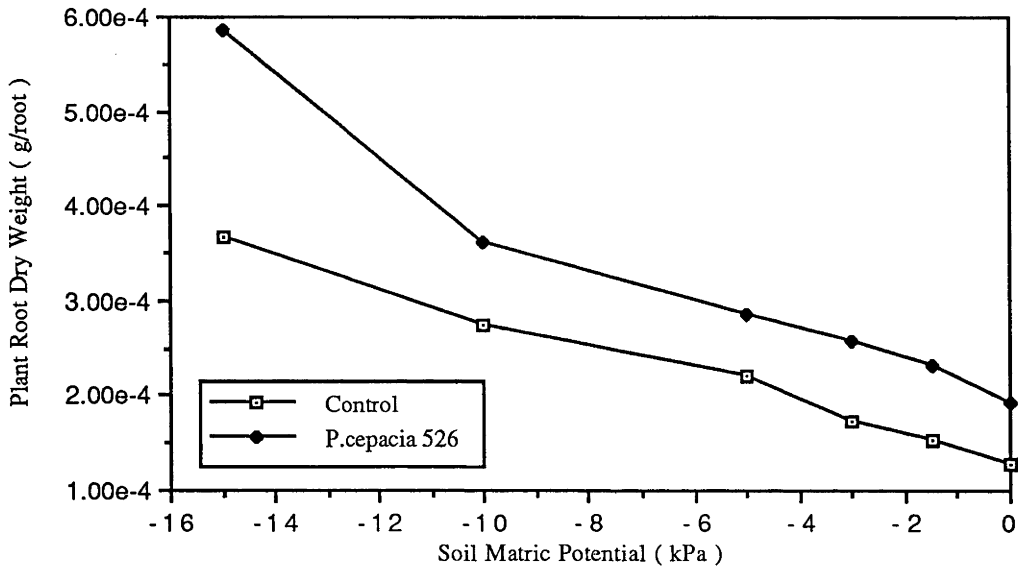
*

Ps. cepacia 10 406.27 0.75 40.63 1.84

Residual 72 1589.76 2.95 22.08

Total 107 53954.95 100 504.25

SE=4.699, CV%=13.6

Fig. 3.9b. Effect of *Pseudomonas cepacia* 526 on plant root weight in the glasshouse.

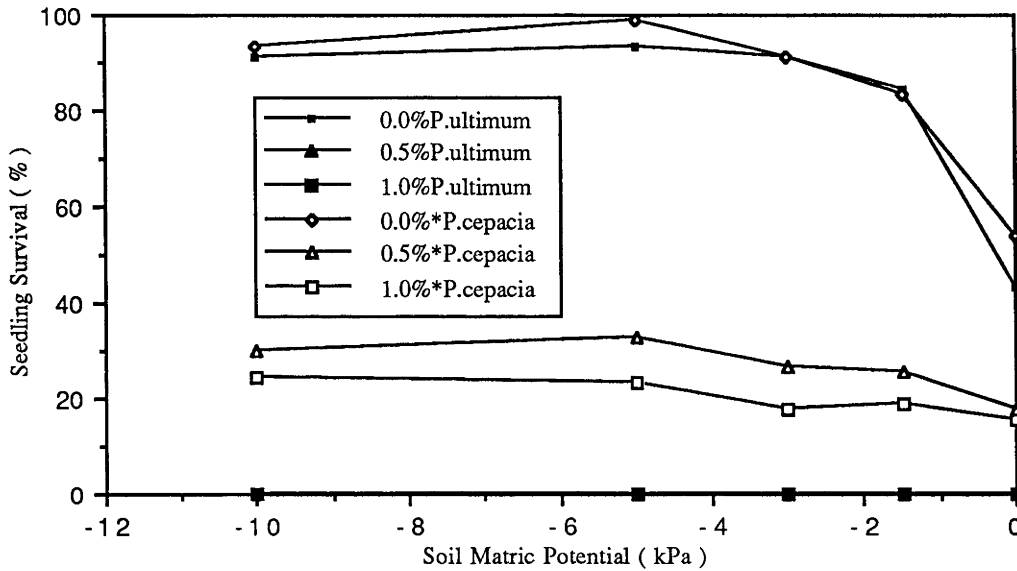
*****ANALYSIS OF VARIANCE*****

Variate: ln(plant root weight)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	5	5.02142	63.77	1.00428	19.99	3.90 (P=0.01)
<i>Ps. cepacia</i>	1	1.24040	15.75	1.24040	24.69	7.82 (P=0.05)
Potential* <i>Ps. cepacia</i>	5	0.40694	5.17	0.08139	1.62	
Residual	24	1.20539	15.31	0.05023		
Total	35	7.87415	100	0.22498		

SE=0.2745, CV%=2.9

Fig. 3.10. Effect of *Pythium ultimum* and *Pseudomonas cepacia* 526 on turnip seedling survival in the constant temperature room



*****ANALYSIS OF VARIANCE*****

Variate: \sin^{-1} (square root of seedling survival rate)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	4	9663.75	13.37	2415.94	79.50	3.65 (P=0.01)
<i>Py. ultimum</i>	2	49754.04	68.83	24877.02	818.59	4.98 (P=0.01)
<i>Ps. cepacia</i>	1	7348.51	10.17	7348.51	241.81	7.08 (P=0.01)
Potential* <i>Py. ultimum</i>	8	320.23	0.44	40.03	1.32	
Potential* <i>Ps. cepacia</i>	4	283.43	0.39	70.86	2.33	
<i>Py. ultimum</i> * <i>Ps. cepacia</i>	2	2851.80	3.95	1425.90	46.92	4.98 (P=0.01)

Potential

*

Py. ultimum

*

Ps. cepacia

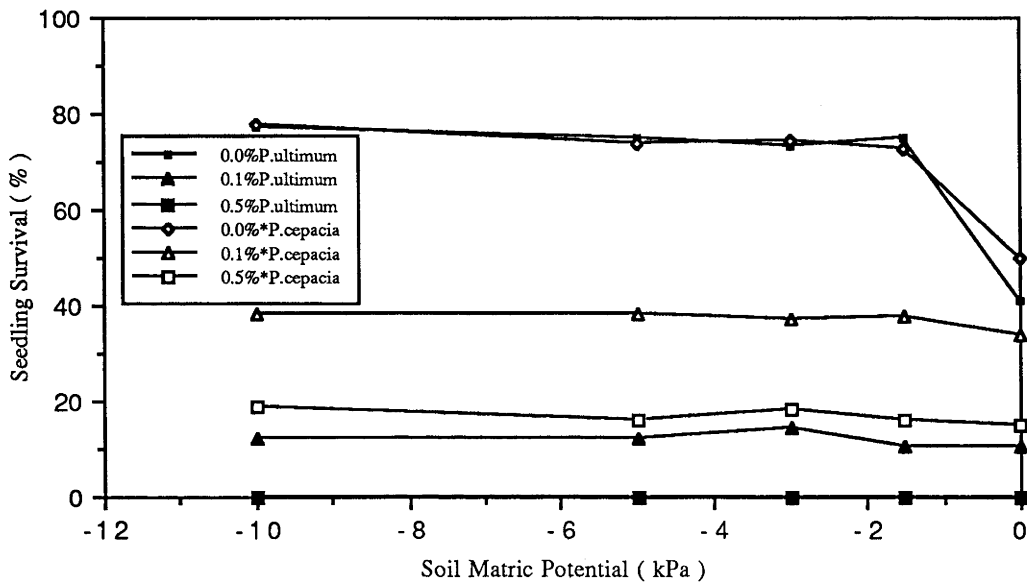
8	239.25	0.33	29.91	0.97
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Residual	60	1823.48	2.52	30.39
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Total	89	72284.49	100	812.19
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SE=5.513, CV%=18.9

Fig. 3.11a. Effect of *Pythium ultimum* and *Pseudomonas cepacia* 526 on turnip seedling survival in the constant temperature room



*****ANALYSIS OF VARIANCE*****

Variate: \sin^{-1} (square root of seedling survival rate)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	4	2422.88	3.53	605.72	21.26	3.65 (P=0.01)
<i>Py. ultimum</i>	2	54612.75	79.42	27306.38	958.45	4.98 (P=0.01)
<i>Ps. cepacia</i>	1	6237.82	9.07	6237.82	218.95	7.08 (P=0.01)
Potential* <i>Py. ultimum</i>	8	419.39	0.61	52.42	1.84	
Potential* <i>Ps. cepacia</i>	4	96.45	0.14	24.11	0.85	
<i>Py. ultimum</i> * <i>Ps. cepacia</i>	2	2828.66	4.11	1414.33	49.64	4.98 (P=0.01)

Potential

*

Py. ultimum

*

Ps. cepacia

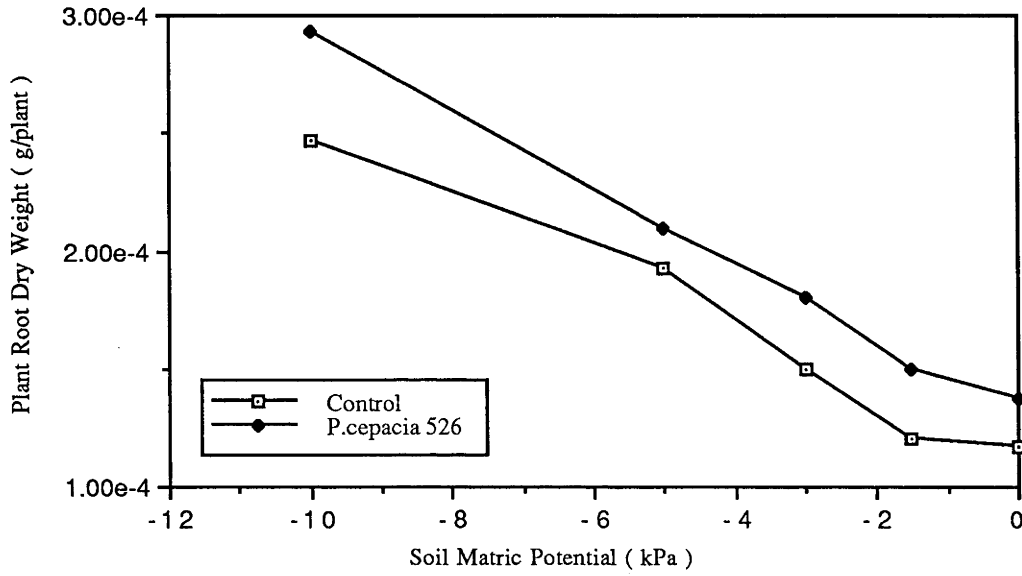
8	433.72	0.63	54.22	1.90
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Residual	60	1709.31	2.49	28.49
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Total	89	68760.98	100	772.60
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SE=5.337, CV%=15.4

Fig. 3.11b. Effect of *Pseudomonas cepacia* 526 on plant root weight in the constant temperature room.



*****ANALYSIS OF VARIANCE*****

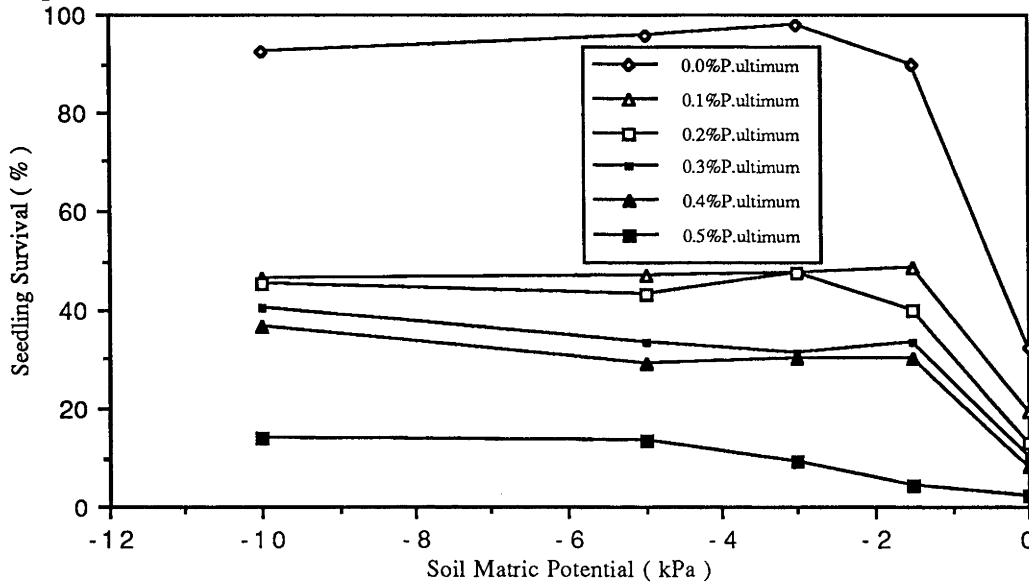
Variate: ln(plant root weight)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	4	1.82472	50.94	0.45618	10.66	4.43 (P=0.01)
<i>Ps. cepacia</i>	1	0.56876	15.88	0.56876	13.29	8.10 (P=0.01)
Potential* <i>Ps. cepacia</i>	4	0.33272	9.29	0.08318	1.94	
Residual	20	0.85604	23.90	0.04280		
Total	29	3.58224	100	0.12353		

SE=0.2069, CV%=2.3

Fig. 3.12. Effect of *Pythium ultimum* on turnip seedling survival in the constant

temperature room



*****ANALYSIS OF VARIANCE*****

Variate: \sin^{-1} (square root of seedling survival rate)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	4	6483.11	18.46	1620.78	16.95	3.65 (P=0.01)
<i>Py. ultimum</i>	5	20907.63	59.53	4181.52	43.74	3.34 (P=0.01)
Potentials* <i>Py. ultimum</i>	20	1994.57	5.68	99.73	1.04	
Residual	60	5735.98	16.33	95.60		
Total	89	35121.29	100	394.62		

SE=6.753, CV%=17.9

3.3.3. The pathogenicity of *Ph. cinnamomi* and the growth-promoting effects of *Ps. cepacia* 526 on the host plant.

In the glasshouse, *Ph. cinnamomi* caused damping-off of turnip (Fig. 3.5a) and reduced the plant weight (Fig. 3.5b) when the soil matric potential was 0 kPa. It neither caused severe disease nor reduced the plant weight when soil matric potential was lower than 0 kPa. However, the fungus significantly reduced the plant root weight when soil matric potential was between 0 and -5.0 kPa (Fig. 3.5c). There were no significant difference between the two inoculum levels of the fungus.

In the constant temperature room, however, *Ph. cinnamomi* caused damping-off (Fig. 3.6a), reduced plant weight (Fig. 3.6b) and plant root weight (Fig. 3.6c) at all the matric potentials tested. Statistical analysis showed that there were no interactions between *Ph. cinnamomi* and soil matric potentials, indicating that such *Ph. cinnamomi* effects were not affected by the soil matric potentials tested. Statistical analysis also showed that there were no significant difference between the two *Ph. cinnamomi* inoculum levels (Fig. 3.6a, 3.6b & 3.6c). When this experiment was repeated with the Buchner funnels covered with plastic food wrap to prevent heterogeneity of water distribution through evaporation (experiment 2), similar results of disease incidences were obtained (Fig. 3.7). There was no difference in the soil water contents between the covered Buchner funnels and the uncovered ones.

In the experiment conducted in the constant temperature room with 6 inoculum levels of *Ph. cinnamomi* (0, 0.2, 0.4, 0.6, 0.8, 1.0%) without *Ps. cepacia* 526 (experiment 3), *Ph. cinnamomi* again caused damping-off (Fig. 3.8a), reduced the plant weight (Fig. 3.8b) and the plant root weight (Fig. 3.8c) without influences from the matric potentials tested. The higher the inoculum

potentials, the significantly lower the seedling survival, the plant weight and the plant root weight (Figures 3.8a, 3.8b & 3.8c).

In independent experiments conducted both in the glasshouse and the constant temperature room with *Ph. cinnamomi* and *Ps. cepacia* 526, ~~The~~ ^{the} bacterium failed to control the damping-off caused by the fungus. However, it consistently increased the seedling survival at 0 kPa (Figures 3.5a, 3.6a & 3.7). This effect was not affected by the presence of *Ph. cinnamomi*.

Ps. cepacia 526 also directly influenced the host plant by significantly increasing the plant root growth (Figures 3.5c & 3.6c), although it did not significantly increase the plant weight (Figures 3.5b & 3.6b). Statistical analysis showed that these effects of *Ps. cepacia* 526 were not affected by either *Ph. cinnamomi* or the soil matric potentials tested.

3.3.4. The pathogenicity of *Py. ultimum* and the plant growth promoting and disease ~~biological~~ control effects of *Ps. cepacia* 526.

In the glasshouse, *Py. ultimum* caused severe disease at all soil matric potentials tested (Fig. 3.9a). There were no significant differences either between the soil matric potentials or between the two fungal inoculum potentials (1.0 & 1.5%).

In the constant temperature room, *Py. ultimum* killed all the plant seeds and seedlings at all soil matric potentials tested (Fig. 3.10). There were no significant difference between the two inoculum potentials (0.5 & 1.0%). When experiment 5 was conducted with lower inoculum potentials (0.1 & 0.5%) and with the Buchner funnels covered with food wrap to prevent the heterogeneity of water distribution through evaporation, similar results were obtained (Fig. 3.11a). However, *Py. ultimum* used at 0.5% caused more severe damping-off than at 0.1%.

In the experiment conducted in the constant temperature room with six inoculum levels of *Py. ultimum* (0, 0.1, 0.2, 0.3, 0.4, 0.5%) without *Ps. cepacia* 526, *Py. ultimum* caused severe disease at all soil matric potentials. The disease became significantly more severe as the inoculum potential increased (Fig. 3.12).

In independent experiments conducted both in the glasshouse and the constant temperature room with *Py. ultimum* and *Ps. cepacia* 526, there was a strong and consistent interaction between *Py. ultimum* and *Ps. cepacia* 526 in regard to the host plant seedling survival. While *Py. ultimum* caused severe damping-off of turnip, *Ps. cepacia* 526 effectively reduced the disease at all soil matric potentials tested (Figures 3.9a, 3.10 & 3.11a). Such disease control by *Ps. cepacia* was not significantly affected by soil matric potentials within the range tested. Apart from its disease control effects, *Ps. cepacia* 526 also increased the seedling survival at 0 kPa in the control funnels (Figures 3.9a, 3.10 & 3.11a).

The effects of *Py. ultimum* on the plant weight and the plant root weight were not determined because too few or no plants survived. However, examination of seedlings collected from funnels without *Py. ultimum* showed that *Ps. cepacia* 526 had increased the plant root weight significantly in the glasshouse (Fig. 3.9b) and in the constant temperature room (Fig. 3.11b).

3.3.5. Recovery of fungi from the diseased plants.

Both *Ph. cinnamomi* and *Py. ultimum* were regularly isolated from diseased turnip seedlings grown in the infested soil but rarely from that grown in the control soil.

3.4. Discussion and Conclusion.

Soil-borne disease is affected by many soil environmental factors. These soil environmental factors have multifaceted effects on the host plant susceptibility, on the pathogen virulence, and on the soil microflora which in

turn can affect the host plant or the pathogen. Their combined effects decide the disease incidence. Thus, conclusion on the role of any soil environmental factors cannot be made without taking into account the specific soil characteristics, the host plant characteristics and the specificity of the pathogen.

Experiments in this chapter involved the host plant, the pathogen, *Ps. cepacia* 526, the air temperature, soil temperature and soil matric potential. Theoretically, these factors could interact with one another in many ways in their effects on plant growth and disease incidences.

3.4.1. The effects of soil matric potential and the soil temperature on the host plant.

Soil matric potential had direct and independent effects on the host plant. The seedling survival rate was significantly smaller in saturated soil, the plant weight also became larger as soil matric potential decreased, indicating that high soil matric potential greatly stressed the plant growth, probably through reduced soil aeration (Drew & Lynch 1980, Griffin 1981). Consequently, this stress could affect the disease incidence. The increase in plant root weight as soil matric potential decreased was physiologically appropriate, because a larger root system would absorb more water at lower matric potentials.

3.4.2. The pathogenicities of *Ph. cinnamomi* and *Py. ultimum* and their relationship with the soil matric potential and the soil temperature.

The associations of soil moisture, especially soil matric potential, and temperature with the incidence of diseases caused by *Ph. cinnamomi* and *Py. ultimum* have been extensively studied and widely recognized (refer to 1.2). However, the effects of soil matric potential and temperature on the disease incidence cannot be generalized, as the mechanisms underlying such effects may be very different in various pathosystem. This is proved by the different results obtained, and the multifaceted explanations given, by different

researchers when they conducted experiments to determine how soil matric potential and temperature imposed their effects using different host plants, plants with different susceptibility, and different soils.

In this study, *Ph. cinnamomi* in the glasshouse caused damping-off and reduced the plant weight only at 0 kPa, and reduced the plant root weight at soil matric potentials between 0 and -5.0 kPa, but the same fungus in the constant temperature room caused damping-off, reduced the plant weight and the plant root weight at all soil matric potentials tested. In the constant temperature room, the daily soil temperatures ranged from 23.5°C to 26°C, which is also the optimum temperature range for *Ph. cinnamomi* (Zentmyer & Marshall 1959, Zentmyer 1980). In the glasshouse, the daily soil temperatures were high at low matric potentials. It is therefore likely that the the soil temperatures in the room favoured *Ph. cinnamomi* and consequently increased the disease. Under such temperatures, *Ph. cinnamomi* was pathogenic at all soil matric potentials tested, even if its inoculum potential was as low as 0.2%. In the glasshouse, the increases in soil temperatures at matric potentials below -5.0 kPa were unfavorable to *Ph. cinnamomi*. Only at potentials higher than -5.0 kPa were soil temperature rises sufficiently reduced so as to permit pathogenic activity by *Ph. cinnamomi* to cause seedling death (0 kPa) or reduce plant root growth (0 to -1.5 kPa). The high soil temperatures may have inactivated the pathogen.

Soil temperature reflects the balance between the heat energy gains and losses (Armson 1977). Solar radiation provides the primary heat energy which can be reflected by the air temperature. Part of the energy is radiated back by the soil surface, part is used in water evaporation, and part is received by the soil resulting in temperature rise.

Soil moisture content exerts a great influence on the soil temperature regime (Armson 1977). Volumetric heat capacity, which is the quantity of heat

required to raise the temperature by 1°C of 1 cm^3 soil, is an important thermal property. The volumetric heat capacity of water is higher than that of most soil solids and greatly exceeds that of a unit volume of soil when gases fill the voids. The volumetric heat capacity of a soil therefore increases with increase in moisture content. The rate of temperature change in soil of high water content is therefore less than in dry soil. Another influence of soil moisture originates through the cooling caused by evaporation. The rate of evaporation is largely controlled by differences in water potentials, temperatures and tortuosity of pathways between a source and a sink for water.

These complex interactions between air temperature, soil moisture and soil temperature were particularly evident in the results presented in this chapter. The daily soil temperature were higher than the air temperature at low matric potentials (especially at -10.0 and -15.0 kPa) but lower at high matric potentials (0 and -1.5 kPa) in the high temperature glasshouse, whereas those in the constant temperature room were unchanged because the daily air temperature change is small. It is likely that these differences in temperatures of soils at various matric potentials arose mainly from differences in soil volumetric heat capacity (due to differences in soil matric potentials) rather than from evaporation, because the differences in soil matric potentials, temperatures and tortuosity of pathways were small.

The results in this chapter suggested great importance of the complex interactions between air temperature, soil matric potential and soil temperature in determining the disease incidence caused by *Ph. cinnamomi*. It is therefore important that, in interpretation of results obtained from experiments where soil moisture is the only variable, attention must also be given to the soil temperature changes which may exist apart from the air temperature. A comparable situation to that existing in my own experiments may have existed in work by

Weste and her colleagues, in which they summarized that during the hot summer, the low soil matric potentials restricted the mycelial growth and sporangial production by *Ph. cinnamomi* (Weste & Taylor 1971, Weste & Vithanage 1977).

The results obtained in this chapter were coincident with those obtained by Hine *et al.* (1964) who showed that the root and heart rot of pineapple caused by *Ph. cinnamomi* in Hawaii were severe between 19°C and 25°C, but disease developed slowly at 30°C and not at all at 36°C.

It should be noted that the water evaporation which may have existed in these experiments did not cause any significant heterogeneity of soil water distribution or reduce soil water content, because the water levels in the reservoirs were constantly kept the same so that the heights of water columns were not changed. This was supported by that there was no difference in the soil water content at the same matric potential between the glasshouse and the constant temperature room.

Plant growth was significantly stressed by soil saturation. This may be an aspect of importance in disease incidence caused by *Ph. cinnamomi* in saturated soil. It may be possible that when the higher soil temperature in the glasshouse was less favorable for *Ph. cinnamomi*, the more predisposition, caused by soil saturation, of the host plant to attack by the fungus became important. When the soil temperature in the room was favorable for the fungus, the predisposing effect of high soil matric potential on the host plant became unimportant. *Ph. cinnamomi* was able to infect the host plant and cause severe disease, even if its inoculum potential was as low as 0.2%.

In this study, *Py. ultimum* showed its pathogenicity at all matric potentials studied in both the glasshouse and the constant temperature room, even if its inoculum potential was as low as 0.1%. Therefore, within the range of

the soil matric potentials and the soil temperatures tested, the disease incidence caused by the *Py. ultimum* isolate was not affected. The results agreed with an early report by Griffin (1963), who found that a range of soil matric potentials (-0.1 to -40 kPa) had no major effect on hyphal growth and sexual reproduction of *Py. ultimum* in artificial soils.

Under suitable soil temperature and soil water conditions, disease severity was associated with the inoculum potential over a certain range, of both pathogens. However, when the inoculum potential of the pathogen reached a certain level, that is 0.5% for *Py. ultimum*, 1.0% for *Ph. cinnamomi*, further increases had no effect.

3.4.3. Plant growth-promotion and biological control of disease by *Ps. cepacia* 526 and the relationships with soil temperatures and matric potentials.

Ps. cepacia exists in soils and on plant root samples as a broad spectrum antagonist to plant pathogens (Spurr & Sasser 1982). Although some work has been done, the mechanisms by which *Ps. cepacia* functions as a biological control agent are uncertain. The relationship between biological control by *Ps. cepacia* and soil matric potential has not previously been studied.

In this study, *Ps. cepacia* 526 promoted plant growth and increased the ability of the host plant to survive under conditions of soil saturation in both the high temperature glasshouse and the constant temperature room experiments. The mechanisms for these effects are unknown. Note however that the underlying effect on the measure of seedling survival cannot be assumed to be operative only at soil saturation. At other matric potentials, it could not be expressed because survival was already close to 100%. This is supported by the data showing that the seedling survivals were not significantly different among the various non-saturated soils in the control.

These experiments also demonstrated a biological control effect of *Ps. cepacia* 526 on the disease incidence. *Ps. cepacia* 526 failed to reduce the damping-off caused by *Ph. cinnamomi* but effectively reduced that caused by *Py. ultimum* at all soil matric potentials in both the high temperature glasshouse and medium constant temperature room. It therefore seemed that a strong and effective antagonistic effect of *Ps. cepacia* 526 existed in the soil against *Py. ultimum* but not against *Ph. cinnamomi*. Complex and different mechanisms of interactions between the bacterium and the fungi in soil were indicated.

In this study, plant-growth-promotion and biological control by *Ps. cepacia* 526 were not affected by either the soil temperature or the soil matric potentials tested. It is therefore suggested that *Ps. cepacia* 526 has good adaptability to a variety of soil temperature and water conditions within the range used. However, this conclusion obviously cannot be extrapolated over different temperature and matric potential ranges other than those used in these experiments.

**CHAPTER 4. ANTAGONISM BY *PSEUDOMONAS CEPACIA* 526
AGAINST *PHYTOPHTHORA CINNAMOMI* AND *PYTHIUM ULTIMUM*
AND THE ASSOCIATION WITH NUTRIENTS**

4.1. Introduction.

In chapter 3, the biological control by *Pseudomonas cepacia* 526 of damping-off of turnip caused by *Phytophthora cinnamomi* and *Pythium ultimum* at various soil temperature and matric potentials was reported. Experiments showed that *Ps. cepacia* 526 effectively controlled disease caused by *Py. ultimum* but not that caused by *Ph. cinnamomi*. However, the preliminary experiments in chapter 2 showed that *Ps. cepacia* 526 was able to cause reduction ~~to~~ⁱⁿ growth of *Ph. cinnamomi* but not of *Py. ultimum*, on PDA ~~agar~~ⁱⁿ ~~medium~~. Experiments in this chapter further examine the antagonistic effects of *Ps. cepacia* strain 526 against *Ph. cinnamomi* and *Py. ultimum* and the influence of various agar and liquid media. Since oat seeds were used in chapter 3 for the preparation of inocula of both *Ph. cinnamomi* and *Py. ultimum*, oatmeal was also used for the culture tests in this chapter.

4.2. Materials and Methods.

4.2.1. *Pseudomonas cepacia* 526 was subcultured on nutrient agar. *Ps. cepacia* 526 suspension was prepared by the method described in 2.2.2 and was used in the following liquid culture tests. *Phytophthora cinnamomi* and *Pythium ultimum* were subcultured on corn meal agar.

4.2.2. Agar plate experiments to evaluate antagonism.

Two experiments were conducted using different agar media.

4.2.2.1. Single medium experiment.

Potato dextrose agar (PDA), corn meal agar (CMA), V-8 agar (V-8), oatmeal agar (OMA), Czapek Dox agar (CDA), nutrient agar (NA) and water agar (WA) were used (see 2.1. for details of media.).

A 2 day old *Ps. cepacia* 526 culture was transferred by a loop onto a 9 cm petri plate with four spots placed evenly round the petri plate, each 5 mm from the edge. The petri plate contained 20 ml agar medium. At the same time, or 2 days later, one fungal plug of either *Ph. cinnamomi* or *Py. ultimum* was transferred from a 7 day old culture onto the center of the petri plate using a sterile 5 mm diameter cork borer and forceps. Control plates were inoculated with fungal plugs only. The petri plates were incubated at 26°C for 1 week. The distance from the edge of the *Ps. cepacia* 526 colony to the edge of the fungal colony was then measured as the size of the fungal growth inhibition zone. There were four replicate plates for each *Ps. cepacia* 526, fungus and medium combination.

4.2.2.2. Mixed media experiment.

This experiment was conducted with agar media made from combined nutrient broth and oatmeal. Nutrient broth concentrations were 0, 0.2 and 0.8% w/v, whereas those of oatmeal were 0, 1 and 4% w/v in distilled water. In each conical flask containing 500 ml distilled water, the desired amounts of nutrient broth and oatmeal and 2% agar were added. The media were heated to boiling with constant agitation, autoclaved at 121°C for 30 min, and then poured into sterile petri plates.

Oatmeal was obtained by: 1) drying oat seeds at 75°C for 2 days; 2) grinding oat seeds and sieving the oatmeal through a 500 micron sieve.

The methods for the inoculation, incubation of the bacterium and the fungi and the measurement of the inhibition zone were the same as in 4.2.2.1,

except that bacterium and fungus were inoculated into the petri plates at the same time.

4.2.3. Liquid media experiments to evaluate antagonism.

4.2.3.1. Growth inhibition of *Ph. cinnamomi* and *Py. ultimum* by *Ps. cepacia* 526 in nutrient suspensions.

This experiment was conducted with nutrient suspensions made from combined nutrient broth and oatmeal. Nutrient broth concentrations were 0, 0.2, 0.4 and 0.8% w/v, whereas oatmeal concentrations were 0, 0.5, 1 and 2% w/v in distilled water. In each conical flask containing 500 ml ~~distilled~~ ^{distilled} water, the desired amounts of nutrient broth and oatmeal were added. The nutrient suspensions were heated to boiling with constant agitation. Each 50 ml of nutrient suspension was then dispensed into a 100 ml conical flask, autoclaved at 121°C for 30 min.

Each conical flask containing 50 ml nutrient suspension was inoculated with a single 7 day old agar plug of *Ph. cinnamomi* using a 5 mm diameter cork borer and forceps. At the same time, 0.5 ml *Ps. cepacia* 526 suspension was added into the conical flask. Control flasks were inoculated with *Ph. cinnamomi* agar plugs only. There were three replicates for each combined nutrient broth and oatmeal suspension. The flasks were placed on a rotary shaker (150 rpm) and maintained at 26°C for 7 days.

After 7 days incubation, the *Ph. cinnamomi* mycelial mat was taken out from each conical flask, rinsed in tap water to remove the residual oatmeal and nutrient broth, dried at 75°C for 4 days and weighed. The mycelial dry weight was used as the measurement of fungal growth. The data were log-transformed and analyzed by analysis of variance (refer to 2.6).

Growth inhibition of *Py. ultimum* by *Ps. cepacia* 526 in the nutrient suspensions was tested in a similar manner in a separate experiment.

4.2.3.2. Growth inhibition of *Ph. cinnamomi* and *Py. ultimum* by *Ps. cepacia* 526 in nutrient suspensions of higher oatmeal concentration.

Two experiments were conducted again with *Ph. cinnamomi* or *Py. ultimum*. However, in these experiments, nutrient broth concentrations were 0 and 0.8% and oatmeal concentrations were 0, 2 and 4% w/v in distilled water. The other procedures were the same as described in 4.2.3.1.

4.2.3.3. Growth inhibition of *Ph. cinnamomi* and *Py. ultimum* by *Ps. cepacia* 526 cell free filtrate (CFF) in nutrient suspensions.

In a 2000 ml conical flask, 1500 ml nutrient broth was inoculated with *Ps. cepacia* 526 by a inoculation loop. The flask was placed on a rotary shaker (150 rpm) and maintained at 26°C for 3 days. The *Ps. cepacia* 526 cell free filtrate (CFF) was obtained by centrifuging the bacterial suspension (5000 g for 30 min at 4°C; Sorvall RC-5B Refrigerated Superspeed Centrifuge) and filtering the supernatant through a 0.2-µm pore size filter (Millipore Corp., Bedford, MA) under sterile condition. The difference in concentration of *Ps. cepacia* 526 CFF before and after the filtration was negligible because there was little change in the volume. This *Ps. cepacia* 526 CFF was used for tests against both *Ph. cinnamomi* and *Py. ultimum*.

Combined nutrient broth and oatmeal suspensions were prepared using methods as described in 4.2.3.1. Nutrient broth was used at 0 and 0.8% and oatmeal was used at 0, 2 and 4% as in 4.2.3.2. Each 50 ml nutrient suspension was dispensed into a 100 ml conical flask, autoclaved at 121°C for 30 min. These flasks were used as control. Nutrient broth and oatmeal suspensions were also prepared in the same way but at double concentrations, and each 25 ml doubly concentrated nutrient suspension was dispensed into a 100 ml conical flask and autoclaved at 121°C for 30 min. These 25 ml doubly concentrated nutrient suspensions in each flask were then each mixed with 25 ml *Ps. cepacia* 526 CFF

under sterile conditions. The final nutrient concentrations in *Ps. cepacia* 526 CFF treated flasks were therefore approximately the same as in the control flasks, as *Ps. cepacia* 526 CFF inevitably brought into the flasks some residual nutrient broth from its original cultures. All the flasks were then inoculated with either a 7 day old agar plug of *Ph. cinnamomi* or a 3 day old agar plug of *Py. ultimum* using a 5 mm diameter cork borer and forceps. The other procedures, including the incubation, measurement of fungal growth and data analysis, were as described in 4.2.3.1. There were three replicates for each combined nutrient broth and oatmeal suspension.

4.3. Results.

4.3.1. Agar plate antagonism experiments.

After having been incubated at 26°C for 1 week, the petri plates were examined for fungal growth inhibition zones caused by *Ps. cepacia* 526 (Tables 4.1 & 4.2). *Ph. cinnamomi* completely overgrew all the control plates in 7 days whereas *Py. ultimum* took 3 days. The growth of *Ph. cinnamomi* was significantly inhibited by *Ps. cepacia* 526 on CMA, CDA, NA, WA, and PDA, but not on OMA nor on V-8 ($P=0.01$, Table 4.1). This inhibition was reduced by the addition and increase in medium oatmeal content (Table 4.2). No clear inhibition zone was shown against *Py. ultimum* on any of the agar media tested. However, when *Py. ultimum* hyphae contacted the bacterial colony, lysis of hyphae occurred, spreading extensively away from the original bacterial colony. *The collapse of hyphae can be assessed visually.*

In the experiment described in 4.2.2.1, *Ps. cepacia* 526 was inoculated into the plates at the same time, or 2 days before the inoculations with fungal plugs. This time difference did not make any difference in the fungal growth inhibition by *Ps. cepacia* 526.

Table 4.1. Fungal growth inhibition zones[#] caused by *Ps. cepacia* 526 on agar plates.

Agar media	<i>Ph. cinnamomi</i>		<i>Py. ultimum</i>	
OMA	0	a	0	a
V-8	0	a	0	a
PDA	6	b	0	a
CMA	16	c	0	a
CDA	17	c	0	a
NA	21	c	0	a
WA	17	c	0	a

[#]Values are means of four replicates representing the distance between the bacterial colony and fungal colony. *Values followed by the same letters are not significantly different according to Duncan's multiple range test (P = 0.01).*

Table 4.2. Fungal growth inhibition zones[#] caused by *Ps. cepacia* 526 on agar plates.

Agar media (Oatmeal*Nutrient Broth)	<i>Ph. cinnamomi</i>		<i>Py. ultimum</i>	
0%*0.0%	16	b	0	a
0%*0.2%	16	b	0	a
0%*0.8%	22	b	0	a
1%*0.0%	2	a	0	a
1%*0.2%	2	a	0	a
1%*0.8%	2	a	0	a
4%*0.0%	0	a	0	a
4%*0.2%	0	a	0	a
4%*0.8%	0	a	0	a

[#]Values are means of four replicates representing the distance between the bacterial colony and fungal colony. *Values followed by the same letters are not significantly different according to Duncan's multiple range test at P = 0.01.*

4.3.2. Growth inhibition of fungi by *Ps. cepacia* 526 in nutrient suspensions.

In the control flasks, the growth of both *Ph. cinnamomi* and *Py. ultimum* was increased by the increases in the ~~medium~~ oatmeal content, but neither fungus responded to increases in the nutrient broth content if the medium also contained oatmeal, indicating that the effect of nutrient broth was negligible if oatmeal was present (Tables 4.3-6). In media without oatmeal, however, the growth responses of *Ph. cinnamomi* and *Py. ultimum* to nutrient broth were different. Increasing the nutrient broth content increased the growth of *Py. ultimum* (Tables 4.3 & 4.4) but not that of *Ph. cinnamomi* (Tables 4.5 & 4.6), although *Ph. cinnamomi* did grow on nutrient agar medium in the agar medium experiments. Thus *Py. ultimum* was able to grow in liquid nutrient broth but *Ph. cinnamomi* was not.

In the presence of *Ps. cepacia* 526, the growth of *Py. ultimum* was completely inhibited. Increasing the contents of oatmeal and nutrient broth in the media did not increase the growth of *Py. ultimum* (Tables 4.3 & 4.4). *Ps. cepacia* 526 also reduced the growth of *Ph. cinnamomi* but this reduction was affected by the ~~medium~~ oatmeal and nutrient broth content. The reduction by *Ps. cepacia* 526 of *Ph. cinnamomi* growth was less when the ~~medium~~ oatmeal content was 2% combined with 0.8% nutrient broth, or when the ~~medium~~ oatmeal content was greater than 2% with or without nutrient broth (Tables 4.5 & 4.6).

4.3.3. Growth inhibition of fungi by *Ps. cepacia* 526 cell free filtrate in nutrient suspensions.

The results of these experiments are shown in tables 4.7 and 4.8. The growth responses of the fungi in the control flasks were same as in 4.3.2.

The growth of *Py. ultimum* was strongly inhibited by *Ps. cepacia* 526 CFF (Table 4.7). *Py. ultimum* grew better in media containing oatmeal or nutrient

Table 4.3. Dry weight means[#] [log(mg)] of *Py. ultimum* mycelium grown in nutrient suspensions in the presence or absence of *Ps. cepacia* 526.

Nutrient suspension (Oatmeal*Nutrient Broth)	<i>Py. ultimum</i>			
	Control		<i>Ps. cepacia</i> 526	
0%*0.0%	1.040 ##	a	1.075	a
0%*0.2%	2.346	bc	1.098	a
0%*0.4%	2.711	cd	1.249	a
0%*0.8%	3.346	d	1.267	a
0.5%*0.0%	4.329	e	1.570	ab
0.5%*0.2%	4.401	ef	1.087	a
0.5%*0.4%	4.706	efg	1.350	a
0.5%*0.8%	4.511	ef	1.209	a
1%*0.0%	4.885	efg	1.372	a
1%*0.2%	4.946	efg	1.509	ab
1%*0.4%	5.272	fgh	1.533	ab
1%*0.8%	5.554	gh	1.824	ab
2%*0.0%	5.823	h	1.386	a
2%*0.2%	5.982	h	1.238	a
2%*0.4%	5.808	h	1.673	ab
2%*0.8%	5.936	h	1.491	ab

#Values are means of three replicates. Values followed by a same letter are not significantly different according to Duncan's multiple range test at P=0.05.

##Weight of *Ph. cinnamomi* agar plugs was from 1.040 to 1.372.

Table 4.4. Dry weight means[#] [log(mg)] of *Py. ultimum* mycelium grown in nutrient suspensions in the presence or absence of *Ps. cepacia* 526.

Nutrient suspension (Oatmeal*Nutrient Broth)	<i>Py. ultimum</i>			
	Control		<i>Ps. cepacia</i> 526	
0%*0.0%	0.622##	a	0.414	a
0%*0.8%	3.348	b	0.875	a
2%*0.0%	5.312	c	0.993	a
2%*0.8%	5.135	c	0.832	a
4%*0.0%	6.050	d	0.581	a
4%*0.8%	6.330	d	0.919	a

#Values are means of three replicates. Values followed by the same letter are not significantly different according to Duncan's multiple range test at P=0.001.

##Weight of *Py. ultimum* agar plugs was from 0.414 to 0.993.

Table 4.5. Dry weight means[#] [log(mg)] of *Ph. cinnamomi* mycelium grown in nutrient suspensions in the presence or absence of *Ps. cepacia* 526.

Nutrient suspension (Oatmeal*Nutrient Broth)	<i>Ph. cinnamomi</i>			
	Control		<i>Ps. cepacia</i> 526	
0%*0.0%	0.556 ^{##}	a	0.903	a
0%*0.2%	0.913	a	0.566	a
0%*0.4%	0.993	a	0.779	a
0%*0.8%	0.811	a	0.586	a
0.5%*0.0%	3.732	bcd	1.040	a
0.5%*0.2%	3.085	bcd	0.934	a
0.5%*0.4%	3.701	bcd	0.982	a
0.5%*0.8%	4.709	de	1.027	a
1%*0.0%	3.498	bcd	0.544	a
1%*0.2%	3.439	bcd	0.497	a
1%*0.4%	4.000	cde	0.928	a
1%*0.8%	4.520	de	1.105	a
2%*0.0%	4.724	de	2.149	abc
2%*0.2%	4.570	de	1.812	ab
2%*0.4%	4.277	de	1.857	ab
2%*0.8%	5.768	e	4.930	de

[#]Values are means of three replicates. Values followed by the same letter are not significantly different according to Duncan's multiple range test at P=0.05.

^{##}Weight of *Ph. cinnamomi* agar plugs was from 0.500 to 1.105.

Table 4.6. Dry weight means[#] [log(mg)] of *Ph. cinnamomi* mycelium grown in nutrient suspensions in the presence or absence of *Ps. cepacia* 526.

Nutrient suspension (Oatmeal*Nutrient Broth)	<i>Ph. cinnamomi</i>			
	Control		<i>Ps. cepacia</i> 526	
0%*0.0%	0.751 ^{##}	a	0.709	a
0%*0.8%	1.001	a	0.980	a
2%*0.0%	5.036	d	0.982	a
2%*0.8%	5.376	d	1.832	b
4%*0.0%	5.599	de	2.667	c
4%*0.8%	6.071	e	5.070	d

[#]Values are means of three replicates. Values followed by the same letter are not significantly different according to Duncan's multiple range test at P=0.001.

^{##}Weight of *Ph. cinnamomi* agar plugs was from 0.700 to 1.001.

Table 4.7. Dry weight means# [log(mg)] of *Py. ultimum* mycelium grown in nutrient suspensions in the presence or absence of *Ps. cepacia* 526 CFF.

Nutrient suspension (Oatmeal*Nutrient Broth)	<i>Py. ultimum</i>			
	Control		<i>Ps. cepacia</i> 526	
0%*0.0%	0.817	a	0.740	a
0%*0.8%	4.080	cd	2.494	b
2%*0.0%	5.482	ef	3.151	bc
2%*0.8%	5.396	ef	2.678	b
4%*0.0%	6.146	f	3.807	bcd
4%*0.8%	6.204	f	3.560	bc

#Values are means of three replicates. Values followed by the same letter are not significantly different according to Duncan's multiple range test at P=0.001.

##Weight of *Ph. cinnamomi* agar plugs was approximately 0.750.

Table 4.8. Dry weight means# [log(mg)] of *Ph. cinnamomi* mycelium grown in nutrient suspensions in the presence or absence of *Ps. cepacia* 526 CFF.

Nutrient suspension (Oatmeal*Nutrient Broth)	<i>Ph. cinnamomi</i>			
	Control		<i>Ps. cepacia</i> 526	
0%*0.0%	0.735	a	0.734	a
0%*0.8%	1.001	a	0.980	a
2%*0.0%	3.888	c	3.388	b
2%*0.8%	3.891	c	3.351	b
4%*0.0%	4.929	d	4.886	d
4%*0.8%	4.967	d	4.847	d

#Values are means of three replicates. Values followed by the same letter are not significantly different according to Duncan's multiple range test at P=0.01.

##Weight of *Ph. cinnamomi* agar plugs was from 0.735 to 1.001.

broth or both than in water, but adding more nutrient did not make any difference to the inhibition of *Py. ultimum* by the bacterial CFF (Table 4.7).

The growth of *Ph. cinnamomi* was inhibited by *Ps. cepacia* 526 CFF (Table 4.8). As in previous experiments, this inhibition was affected by the oatmeal and nutrient broth content of the media. There was inhibition when the oatmeal content was 2% but not at 4%.

4.4. Discussion and Conclusion.

In selecting biological control agents of soil-borne plant pathogens, *in vitro* culture tests are generally an important step as they make it simpler and quicker to do large scale screening of a number of microorganisms. However, the results of the tests are not always matched by performance in practice (Utkhede 1983, Utkhede 1986), as indicated by the conflicting results obtained in the preliminary experiments in chapter 2 and those obtained in chapter 3, due to the great differences between the complex changing soil conditions and the relatively uniform and stable culture conditions. Nutrient availability also usually differs greatly between natural and culture situation. Therefore, caution must be taken in performing *in vitro* culture tests and in interpreting their results. The results would be more convincing if *in vitro* tests were performed under various conditions such as with media differing in their nutrient components and with both agar and liquid media, as in experiments described in this chapter.

Nutrient has been a paramount factor in the interaction between *Ph. cinnamomi* and *Ps. cepacia* 526 in these experiments. On agar media, inhibition of *Ph. cinnamomi* by *Ps. cepacia* 526 was none or reduced on media such as OMA, V-8, PDA and combined nutrient broth and oatmeal agar. These media are relatively rich in nutrients. The first liquid media test showed a significant

reduction of inhibition of *Ph. cinnamomi* by *Ps. cepacia* 526 when combined 0.8% nutrient and 2% oatmeal liquid medium was used. These results indicated a strong nutrient effect. Experiments with higher nutrient levels were therefore performed in the second liquid media experiment and the effects of high nutrient levels were again confirmed.

High nutrient levels could play their roles in either nutrient competition or antibiotic interaction between *Ph. cinnamomi* and *Ps. cepacia* 526. Nutrient competition, however, was unlikely to be important because the growth of *Ph. cinnamomi* in nutrient suspension containing 2% oatmeal was inhibited by *Ps. cepacia* 526 cell free filtrate. The reactions of *Ph. cinnamomi* to *Ps. cepacia* 526 and the cell free filtrate were similar so it is likely that the main effect of nutrient level was on the antibiotic interaction.

One explanation for this nutrient effect on the antibiotic interaction between *Ph. cinnamomi* and *Ps. cepacia* 526 assumes that the effects were on *Ps. cepacia* 526. High nutrient level might reduce the antibiotic production by *Ps. cepacia* 526 resulting in reduced inhibition of *Ph. cinnamomi*. If this were so, we should expect that the cell free filtrate produced from 8% nutrient broth *Ps. cepacia* 526 culture would have the same effect on *Ph. cinnamomi* grown in liquid media of various nutrient concentrations. However, the results obtained with *Ps. cepacia* 526 CFF have showed varied inhibition of *Ph. cinnamomi* (Table 4.8). A second explanation, that the nutrients determined the reaction of *Ph. cinnamomi* to the *Ps. cepacia* 526 antibiotic effects, is therefore likely to be valid. The nutrients may have affected the fungal susceptibility to the bacterial antibiosis or the effect of the fungus on the bacterium.

The nutrient effects on *Ph. cinnamomi* were not exactly the same on agar plates as in liquid media. For example, *Ph. cinnamomi* was only slightly inhibited by *Ps. cepacia* 526 in liquid media containing 1% oatmeal or even 2%

oatmeal (Tables 4.5&4.6), but it was strongly inhibited on agar media containing only 1% oatmeal (Table 4.2). There may therefore be quite different mechanisms involved in the reactions of *Ph. cinnamomi* on agar plates and in liquid media. How *Ph. cinnamomi* reacted to the *Ps. cepacia* 526 antibiotic effects at various nutrient levels needs to be clarified. One explanation is that *Ph. cinnamomi* was able to grow so efficiently at high nutrient levels that its quick growth offset the antibiotic effect of *Ps. cepacia* 526. This was supported in liquid media experiment with *Ps. cepacia* 526 cell free filtrate where the growth of *Ph. cinnamomi* was inhibited by *Ps. cepacia* 526 CFF in nutrient suspension containing 2% oatmeal but not in those containing 4% oatmeal. At high nutrient levels, *Ph. cinnamomi* may also have performed some defensive actions against *Ps. cepacia* 526, for example, production of antibiotics.

Although increases in nutrient level increased the growth of *Py. ultimum* in the absence of *Ps. cepacia* 526, such increases had little influence in reducing the growth inhibition of *Py. ultimum* by *Ps. cepacia* 526. There is no significant evidence of nutrient competition between *Py. ultimum* and *Ps. cepacia* 526, so any interaction is likely to mainly involve antibiosis and this was not affected by nutrient levels.

Bacteria may affect fungi by producing different types of antibiotics. Some are quickly released upon production, resulting in an inhibition zone in agar medium experiment. Some antibiotics, however, are produced within the bacterial cell and are only released when bacterial lysis occurs. Inhibition caused by such antibiotics cannot be reflected by inhibition zones on agar media (Howell & Stipanovic 1979, Howell & Stipanovic 1980). Liquid medium experiments may help to demonstrate the presence of such antibiotics.

In the liquid medium experiments, both *Ps. cepacia* 526 and its CFF caused ~~great~~ growth inhibition of *Py. ultimum*. *Ps. cepacia* 526 caused hyphal ^{greater}

lysis in *Py. ultimum* after direct contact but did not produce inhibition zones in agar media. On the other hand, *Ps. cepacia* 526 and its CFF reduced the growth of *Ph. cinnamomi* in liquid medium and also produced inhibition zones in agar media. It therefore seems likely that *Ps. cepacia* 526 produces at least two antibiotics, one active against *Py. ultimum* being produced inside the bacterial cell and released after its lysis, the other active against *Ph. cinnamomi* being released into agar and liquid media soon after production.

The results of interactions between bacterium and fungi in culture are determined not only by bacterial antibiosis but also by the fungal responses. These again are affected by the medium nutrients. Since there were great difference in the inhibitions of *Ps. cepacia* 526 on *Ph. cinnamomi* and *Py. ultimum*, It is possible that differences existed in the fungal susceptibility to the bacterial antibiosis or the effect of the fungus on the bacterium. This requires further study.

CHAPTER 5. EFFECTS OF *PHYTOPHTHORA CINNAMOMI* AND *PYTHIUM ULTIMUM* ON *PSEUDOMONAS CEPACIA* 526

5.1. Introduction.

The interactions between a plant pathogen and a biological control agent may be in both directions. While the biological control agent may be antagonistic to the plant pathogen, the plant pathogen may also impose a counter-effect on the biological control agent. In the studies of such complex interactions, however, the antagonistic effects of the biological control agent on the plant pathogen are usually the focus and the effects of the plant pathogen on the biological control agent are usually neglected. To achieve successful disease biological control, these two aspects require equal attention.

The experiments in chapters 3 and 4 have already shown that *Phytophthora cinnamomi* and *Pythium ultimum* responded differently both to *Pseudomonas cepacia* 526 and to nutrient concentrations. The results also indicated possible production by the bacterium of two or more antibiotics, with contrasting properties, one specifically affecting each fungus. In ~~experiment of~~ ^{the experiments reported in} this chapter, the effects of pyrrolnitrin, an antibiotic produced by *Ps. cepacia* 526, on *Ph. cinnamomi* and *Py. ultimum* were examined. More experiments were also conducted to further elucidate the reactions of *Ph. cinnamomi* and *Py. ultimum* to *Ps. cepacia* 526.

5.2. Materials and Methods.

5.2.1. *Ps. cepacia* 526, *Ph. cinnamomi* and *Py. ultimum*.

Ps. cepacia 526 was subcultured on nutrient agar. *Ps. cepacia* 526 nutrient broth suspension expected to contain approximately 10^9 colony forming units per milliliter (cfu/ml) was prepared by the method described in 2.2.2. The suspension

was serially diluted to obtain suspensions expected to contain 10^2 , 10^3 , 10^4 cfu/ml. *Ph. cinnamomi* and *Py. ultimum* were subcultured and maintained on corn meal agar.

5.2.2. Agar media.

Agar media were made from combined nutrient broth and oatmeal. In the experiments described in 5.2.3 and 5.2.4.1, nutrient broth concentration was either 0 or 0.8%, and oatmeal concentration was either 0, 1 or 4%. In experiments 5.2.4.2 and 5.2.4.3, nutrient broth concentration was 0.8% while that of oatmeal was 0, 1 or 4%. In each conical flask containing 500 ml distilled water, the desired amount of nutrient broth and oatmeal and 2% agar were added. The medium was heated to boiling with constant agitation, autoclaved at 121°C for 30 min and poured into sterile petri plates in a laminar flow cabinet.

5.2.3. The effects of pyrrolnitrin on mycelial growth of *Ph. cinnamomi* and *Py. ultimum*.

Ph. cinnamomi were grown on various agar media (refer to 5.2.2) for 3 days and *Py. ultimum* for 1 day at 26°C .

Pyrrolnitrin is an antibiotic produced by *Ps. cepacia* 526 (refer to 1.3.2). Since pyrrolnitrin is sensitive to ~~sun light~~ ^{Sunlight} (Arima *et al.* 1965), this experiment was conducted in a room with low intensity fluorescent light. Pyrrolnitrin crystals were provided by Mr. R. Rickards, Research School of Chemistry, ANU. A 200 $\mu\text{g/ml}$ stock solution was made by dissolving 2 mg pyrrolnitrin in 10 ml 50% alcohol. This stock solution was further diluted with 50% alcohol to obtain 100, 50 and 10 $\mu\text{g/ml}$ stock solutions. Conical flasks containing stock solution of various concentration were wrapped with aluminium foil and stored at 4°C .

Blank paper discs (1/4 inch diameter, BBL) were dipped in pyrrolnitrin stock solutions of 200, 100, 50, 10 $\mu\text{g/ml}$ for 5 min, respectively. Each paper disc contained 25 μl solution when saturated. The paper discs were then air dried in

a laminar flow cabinet for 15 min to evaporate the alcohol. After petri plates inoculated with *Ph. cinnamomi* were incubated for 3 days and those with *Py. ultimum* were incubated for 1 day at 26°C, three paper discs dipped in the same pyrrolnitrin stock solution were placed onto each petri plate 1 cm away from fungal colony. Paper discs dipped in both 50% alcohol and distilled water were used as controls. The plates were again incubated at 26°C for 1 week and the size of the fungal growth inhibition zone was measured. There were four replicates for each treatment.

5.2.4. Production of antibiotics by *Ph. cinnamomi*.

5.2.4.1. Movement and reproduction of *Ps. cepacia* 526 along hyphae of *Py. ultimum* and *Ph. cinnamomi*.

In the culture experiments in chapter 4, it was noted that when *Py. ultimum* hyphae contacted the *Ps. cepacia* 526 colony, lysis of hyphae occurred and spread extensively away from the original bacterial colony. This did not occur with *Ph. cinnamomi* hyphae. This experiment set out to further study if *Ps. cepacia* 526 movement occurs along the fungal hyphae.

Agar media described in 5.2.2. were used. Each agar plate was inoculated with a 7 day old fungal plug of either *Ph. cinnamomi* or *Py. ultimum*. The petri plates were incubated at 26°C for 1 day for *Py. ultimum*, 3 days for *Ph. cinnamomi*. A 2 day old *Ps. cepacia* 526 nutrient agar culture was then used to inoculate each petri plate, with four spots directly contacting the fungal colony (Fig. 5.1). The petri plates were again incubated at 26°C and examined daily for the movement of *Ps. cepacia* 526 along the fungal hyphae. This movement was visible because of the fungal hyphal collapse accompanying presence of the bacterium (Fig. 5.1). There were four replicates for each *Ps. cepacia* 526, fungus and medium combination.

When the movement of *Ps. cepacia* 526 along hyphae was visible, a 9 square millimeter piece of agar was cut from the forefront of fungal hyphal collapse. The agar plug was mounted on a metal stub, plunged into liquid nitrogen slush at -230°C to rapidly freeze it. It was then quickly transferred to the preparation chamber of a Hexland CT1000 Cryostage attached to a Cambridge S360 Scanning Electron Microscope (SEM). Coating of the sample with a 10 nanometer layer of gold was carried out in the preparation chamber at -170°C. The specimen was then transferred to the viewing chamber of the SEM, which was kept at -170°C chamber temperature by use of a liquid nitrogen-cooled stage. Observation was carried out for the presence and distribution of bacteria and morphological changes in the fungal hyphae.

5.2.4.2. Inhibition of *Ps. cepacia* 526 on agar media by *Ph. cinnamomi* and *Py. ultimum*.

Since it was found in experiment 5.2.4.1 that *Ps. cepacia* 526 could quickly move and may also have reproduced along the hyphae of *Py. ultimum* but not along that of *Ph. cinnamomi*, it was considered that *Ps. cepacia* 526 may have been chemically inhibited by *Ph. cinnamomi*, but not by *Py. ultimum*. That movement was affected by differences between the hyphal surface of the two fungi was considered less likely because of the taxonomic similarity between them.

This experiment was designed to examine whether *Ph. cinnamomi* and *Py. ultimum* can inhibit *Ps. cepacia* 526 without direct contact with the bacterium. A millipore filter paper disc (1.0 cm diameter, 0.2 µm pore size, Millipore Corp., Bedford, MA) was found suitable for the study in a preliminary experiment. The millipore filter paper disc does not allow either the bacterium or the fungi to grow through but allows the nutrients to pass. When placed on agar

media, the millipore filter paper supported as good bacterial growth as did direct bacterial contact with the agar media.

Agar media described in 5.2.2. were used for this experiment. Each agar plate was inoculated with a 7 day old fungal plug of either *Ph. cinnamomi* or *Py. ultimum*. The petri plates were incubated at 26°C for 1 day for *Py. ultimum*, and 3 days for *Ph. cinnamomi*.

Three millipore filter paper discs were then placed on top of either 1 day old *Py. ultimum* colony or 3 day old *Ph. cinnamomi* colony in each petri plate. *Ps. cepacia* 526 suspensions (0.01 ml) expected to contain 10^2 , 10^3 , 10^4 colony forming units per milliliter were then separately dripped onto the center of each paper disc. Control tests were made by inoculating bacterial suspensions onto paper discs placed on different agar media which had not been inoculated with the fungi. There were four replicates for each *Ps. cepacia* 526, fungus and medium combination. All plates were then incubated at 26°C.

After 2 days incubation, the plates were examined for the numbers of bacterial colonies on each paper disc. All plates were again incubated at 26°C for 5 days. The average diameter of individual bacterial colonies on each paper disc were measured using a stereo microscope and a calibrated micro-ruler. Each paper disc on which bacterial colonies grew was then put into a test tube containing 10 ml distilled water. The bacterial colonies were washed off by vigorously shaking the test tube. The bacterial suspension obtained was serially diluted and plated out on nutrient agar petri plates. The petri plates were then incubated at 26°C for 2 days and the numbers of bacteria counted. The total bacterial population and the average population per colony on each paper disc were then calculated.

To ensure that maximum but countable numbers of colonies were obtained, bacterial suspensions (0.01 ml each) of three concentrations (expected

to contain 10^2 , 10^3 , 10^4 cfu/ml) were used for inoculation onto three paper discs in the same petri plate. However, the numbers of colonies, the average size of individual bacterial colonies and the average bacterial population per colony were recorded only on paper discs that were inoculated with the same bacterial suspension and produced $\approx 10^1$ colonies (usually around 20 colonies) in the controls.

5.2.4.3. Inhibition of *Ps. cepacia* 526 on agar media by *Ph. cinnamomi* metabolites (exudates).

This experiment was to study if *Ph. cinnamomi* exuded antibiotic materials into the agar media and the longevity of any antibiotics so produced.

Various agar media described in 5.2.2. were again used for this test. The pH value of the agar medium in the petri plates were measured with pH litmus papers. A piece of millipore filter paper (4.7 cm diameter, 0.2 μ m pore size, Millipore Corp., Bedford, MA) was placed onto the center of each agar plate. A 7 day old fungal plug of *Ph. cinnamomi* was then inoculated onto the center of the millipore filter paper. After *Ph. cinnamomi* had grown for 3 days at 26°C, without growing over the millipore filter paper and contacting the agar plate, the millipore filter papers together with the fungal colonies were peeled off from the agar plates. *Ps. cepacia* 526 suspensions (0.01 ml) expected to contain 10^2 , 10^3 , 10^4 colony forming units per milliliter were then separately spotted onto the area where *Ph. cinnamomi* had grown on the plate. The pH value of the agar where *Ph. cinnamomi* had grown was measured again. Control tests were made by dripping bacterial suspensions onto different agar media with no previous *Ph. cinnamomi* growth. There were four replicates in this test.

The numbers of bacterial colonies and the average size of individual bacterial colonies at each spot were again recorded as described in the previous

section. The bacterial population per colony was not measured as it can be inferred from the size of individual bacterial colonies.

5.2.4.4. The association of nutrients with *Ph. cinnamomi* antibiotic production.

This experiment was conducted to evaluate the effects of various nutrient components on *Ph. cinnamomi* antibiotic production.

Five agar media used in this experiment were as follows: 1) 0.8% nutrient broth (pH 6.8); 2) 0.8% nutrient broth with 1% D-glucose (pH 6.7); 3) 8 g nutrient broth solids with 1.035 g KNO₃ per liter (pH 6.7); 4) 8 g nutrient broth solids, 10 g D-glucose and 1.035 g KNO₃ per liter (pH 6.7); and 5) 8 g nutrient broth solids, 10 g D-glucose, 1.035 g KNO₃ and 100 µg thiamine hydrochloride per liter (pH 6.7).

The procedures of this experiment were as described in 5.2.4.2.

5.2.4.5. Extraction and bioassay of possible antibiotics from *Ph. cinnamomi* liquid culture.

As inhibition of *Ps. cepacia* 526 by *Ph. cinnamomi* was observed in the previous experiments, attempts were made to isolate the possible antibiotics from *Ph. cinnamomi* liquid culture.

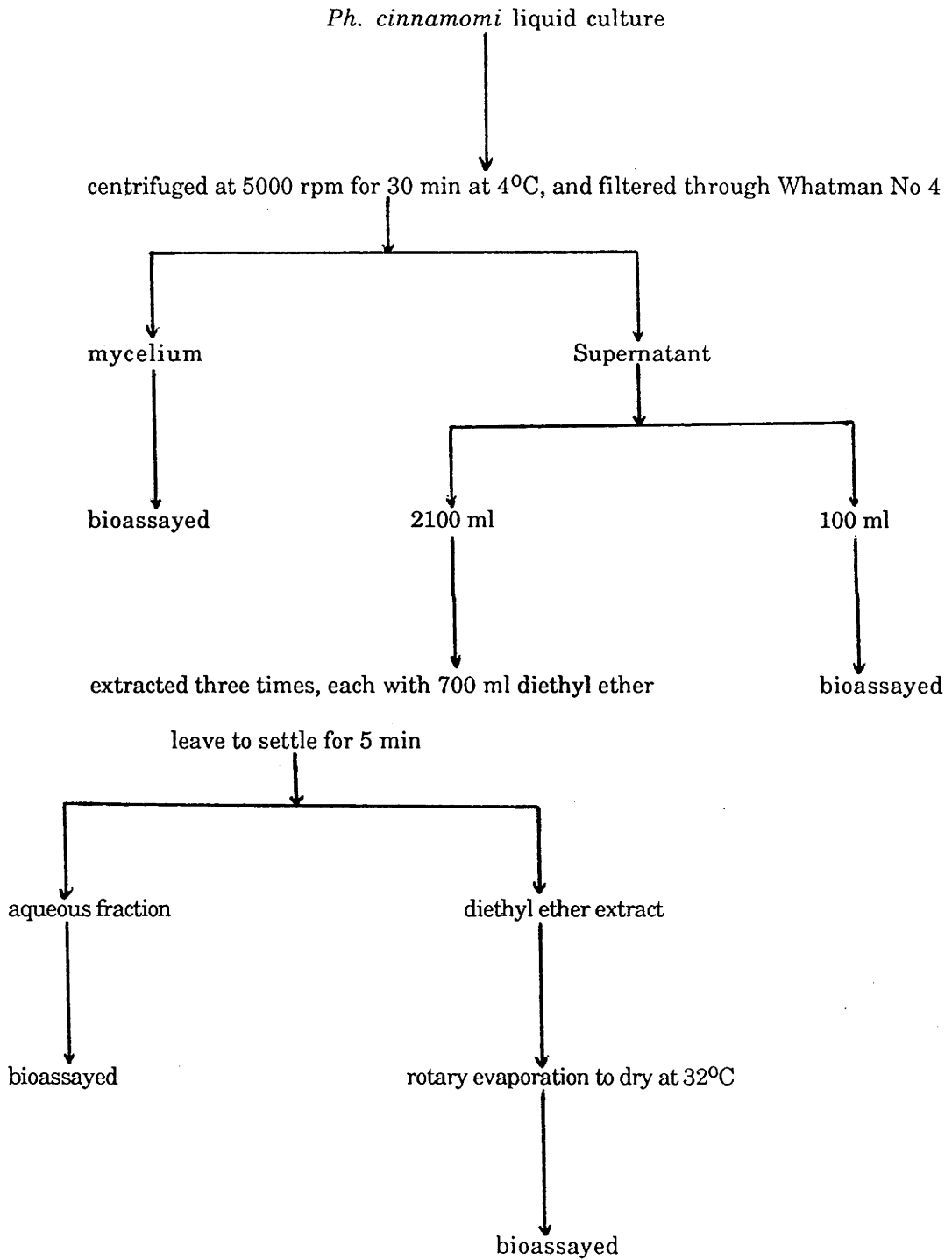
5.2.4.5.1. Culture of *Ph. cinnamomi*:

The liquid medium consisted of 0.8% nutrient broth and 2% oatmeal, autoclaved at 121°C for 30 min.

Five 2000 ml conical flasks, each containing 500 ml liquid medium, were each inoculated with three 7 day old 5 mm diameter agar plugs of *Ph. cinnamomi*. The flasks were placed on a rotary shaker (150 rpm) and maintained at 26°C for 7 days. The liquid cultures were collected for extraction of antibiotics.

5.2.4.5.2. Extraction of antibiotics.

The procedures are described as follows:



5.2.4.5.3. Bioassays.

The antibiotic effects against *Ps. cepacia* 526 of (a). the *Ph. cinnamomi* mycelium; (b). the supernatant of the liquid culture; (c). the aqueous fraction and (d). the diethyl ether extract were tested through a series of bioassays.

Ph. cinnamomi mycelium was collected in a conical flask and killed by adding 75% ethanol and leaving overnight. The dead mycelium was then picked out, air dried to evaporate the ethanol and spread sparsely on nutrient agar plates. Millipore filter paper disc (1.0 cm diameter, 0.2 μ m pore size, Millipore Corp., Bedford, MA) were then placed onto the mycelium and 0.01 ml *Ps. cepacia* 526 suspensions expected to contain 10^2 , 10^3 , 10^4 colony forming units per milliliter were spotted onto each paper disc. The ethanol used to kill the mycelium was also tested for possible extraction of antibiotic materials from the mycelium.

The methods for the bioassay of the ethanol used to kill the mycelium, the supernatant of the liquid culture, the aqueous fraction and the diethyl ether extract were the same. The supernatant of the liquid culture was filtered through a 0.2- μ m millipore filter before being tested. The dry diethyl ether extract was dissolved in 21 ml diethyl ether. The diethyl ether solution was then further diluted with diethyl ether to obtain solutions as if being obtained by dissolving the dry diethyl ether extract in 210 ml or 2100 ml diethyl ether. The diethyl ether extract was thus tested at three concentrations equivalent to the original liquid culture, 10 times and 100 times concentrated solutions.

Millipore filter paper disc (1.0 cm diameter, 0.2 μ m pore size, Millipore Corp., Bedford, MA) were placed into the ethanol, the supernatant of the liquid culture, the aqueous fraction or the diethyl ether extract solutions for 1 min. They were then air dried to evaporate the ethanol, water or diethyl ether before

being placed on either nutrient agar plates or agar plates containing 0.8% nutrient broth and 2% oatmeal. *Ps. cepacia* 526 suspensions (0.01 ml) expected to contain 10^2 , 10^3 , 10^4 colony forming units per milliliter were then spotted onto each paper disc.

For all bioassays, control tests were made by using sterile distilled water, 75% ethanol or diethyl ether, where applicable. After *Ps. cepacia* 526 was inoculated onto the millipore filter paper discs, all nutrient agar plates were incubated at 26°C. The numbers of bacterial colonies and the average size of the individual colonies were recorded as described in 5.2.4.2.

5.2.4.6. Extraction of antibiotics from *Ph. cinnamomi* and *Ps. cepacia* 526 mixed liquid culture and from *Ps. cepacia* 526 liquid culture and their bioassays.

This experiment was to examine if *Ph. cinnamomi* require the presence of *Ps. cepacia* 526 to induce its antibiotic production in liquid culture.

5.2.4.6.1. Culture of *Ph. cinnamomi* and *Ps. cepacia* 526:

The liquid medium consisting of 0.8% nutrient broth and 2% oatmeal was again used.

Five 2000 ml conical flasks, each containing 500 ml liquid medium, were each inoculated with three 7 day old agar plugs of *Ph. cinnamomi*. At the same time, 1 ml *Ps. cepacia* 526 suspension (approximately 10^9 cfu/ml) was added into each conical flask. Five control flasks were inoculated with *Ps. cepacia* 526 only. The flasks were placed on a rotary shaker (150 rpm) and maintained at 26°C for 7 days. The liquid cultures were collected for extraction of antibiotics.

5.2.4.6.2. Extraction of antibiotics.

The *Ph. cinnamomi* and *Ps. cepacia* 526 mixed liquid culture and the *Ps. cepacia* 526 liquid culture were collected separately for the extractions of antibiotics. The procedures for the extractions of antibiotics were exactly the same as described in 5.2.4.4.2.

5.2.4.6.3. Bioassays.

The antibiotic effects against *Ps. cepacia* 526 of (a). the filtered *Ph. cinnamomi* and *Ps. cepacia* 526 mixed liquid culture; (b). the diethyl ether extract of the *Ph. cinnamomi* and *Ps. cepacia* 526 mixed liquid culture; (c). the filtered *Ps. cepacia* 526 liquid culture; and (d). the diethyl ether extract of the *Ps. cepacia* 526 liquid culture were tested. The methods for the bioassays were same as in 5.2.4.5.3, except that only the 100 times concentrated diethyl extracts were tested. Controls used sterile distilled water and diethyl ether.

5.2.4.7. Inhibition of *Ps. cepacia* 526 in liquid culture by *Ph. cinnamomi*.

This experiment was to study if *Ps. cepacia* 526 was inhibited by *Ph. cinnamomi* in liquid culture and the effect of nutrient composition.

Three liquid media were used for this test, i.e. 0.8% nutrient broth; 0.8% nutrient broth with 1% oatmeal; and 0.8% nutrient broth and 2% oatmeal. In each conical flask containing 500 ml distilled water, desired amounts of nutrient broth and oatmeal were added. The nutrient suspensions were heated to boiling with constant agitation. Each 50 ml nutrient suspension was then dispensed into a 100 ml conical flask, autoclaved at 121°C for 30 min. The pH values of different liquid media were measured before autoclaving.

Each conical flask containing 50 ml nutrient suspension was inoculated with a 7 day old agar plug of *Ph. cinnamomi*. At the same time, 0.5 ml *Ps. cepacia* 526 of a 10^9 cfu/ml broth suspension was added into the conical flask. Control flasks were inoculated with 0.5 ml *Ps. cepacia* 526 suspension only. There were three replicates for each combined nutrient broth and oatmeal suspension. The flasks were placed on a rotary shaker (150 rpm) and maintained at 26°C for 7 days.

After 7 days, the *Ps. cepacia* 526 populations in various liquid media with or without *Ph. cinnamomi* were counted by the serial dilution method. The pH values of the different liquid cultures were measured again.

5.3. Results.

5.3.1. The effects of pyrrolnitrin on mycelial growth of *Ph. cinnamomi* and *Py. ultimum*.

The plates were incubated at 26°C for a week and checked daily for the size of the fungal growth inhibition zone. Both *Ph. cinnamomi* and *Py. ultimum* grew around the paper discs dipped in pyrrolnitrin solutions as they did around the paper discs dipped in both 50% alcohol and distilled water control, with no growth inhibition zone existing. Pyrrolnitrin tested at 200, 100, 50, 10 µg/ml did not inhibit mycelial growth of either fungus.

5.3.2. Movement and reproduction of *Ps. cepacia* 526 along hyphae of *Py. ultimum* and *Ph. cinnamomi*.

It was noted that *Ps. cepacia* 526 quickly moved along the hyphae of *Py. ultimum* on all agar media tested (Fig. 5.1), with a average of 3 mm/day, but no bacterial movement occurred along hyphae of *Ph. cinnamomi*.

Scanning electron microscope observations (Fig. 5.2) showed that *Ps. cepacia* 526 were present abundantly on hyphae of *Py. ultimum*. Infected *Py. ultimum* hyphae became deformed or distorted as bacteria contacted them. In areas where movement had already occurred, *Ps. cepacia* 526 were so abundant that the hyphae of *Py. ultimum* were overgrown by the bacterium and could not be seen. *Py. ultimum* hyphae in the control plates and *Py. ultimum* hyphae cut from the uninfected area in the *Ps. cepacia* 526 inoculated plates showed normal hyphal growth, with no bacterium present on them. These evidences again indicated that the bacterium has moved and had almost certainly reproduced

Fig. 5.1. Movement of *Ps. cepacia* 526 along *Py. ultimum* hyphae.

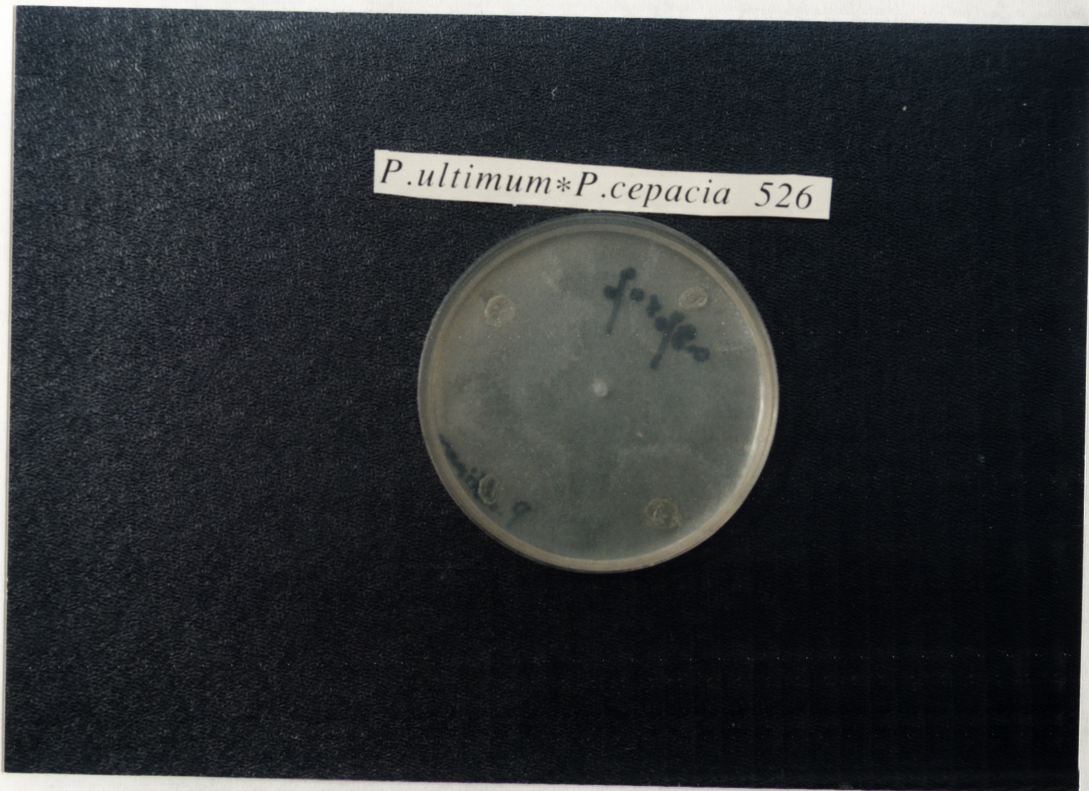
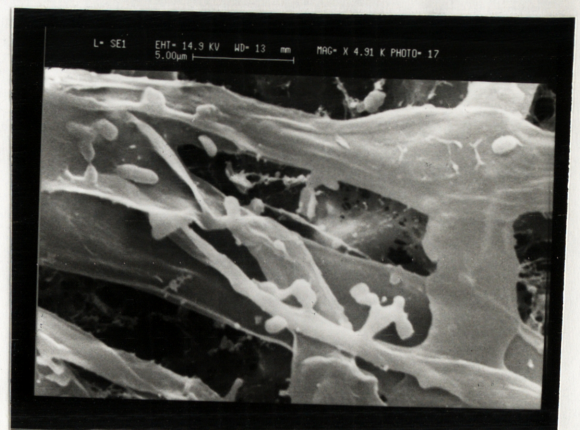
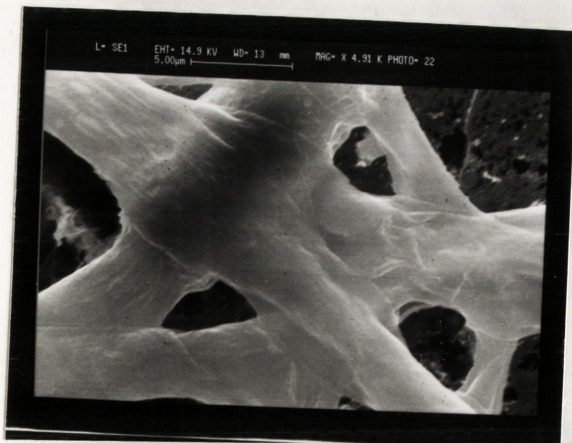
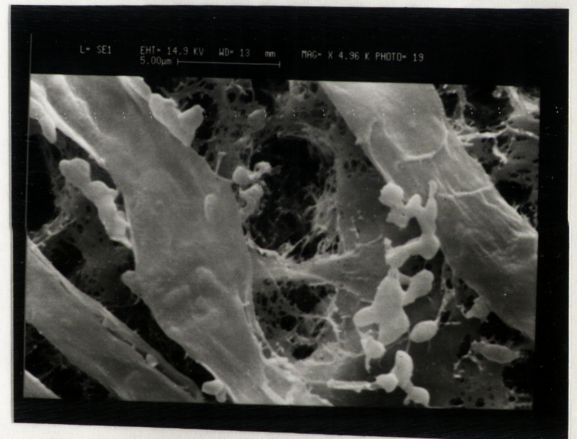
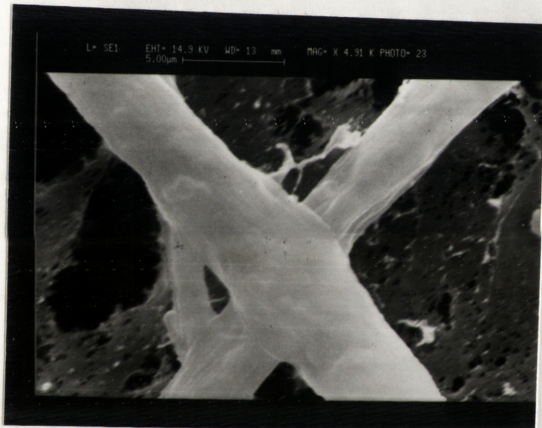
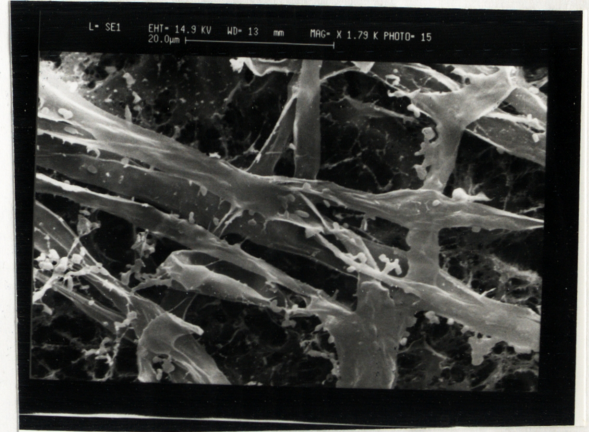
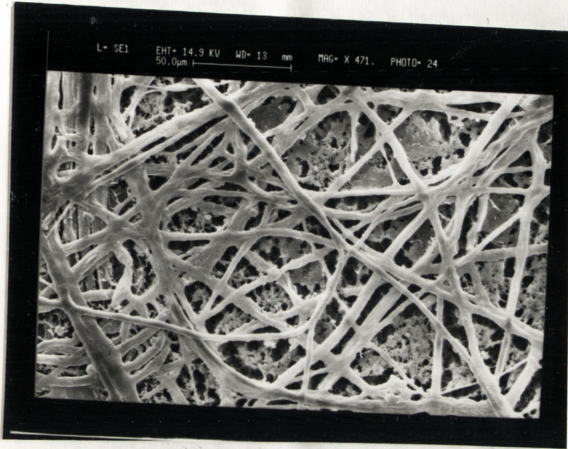


Fig. 5.2. *Ps. cepacia* 526 was abundantly present along *Py. ultimum* hyphae. Photos on left are of control plates and those on right are of inoculated plates.



along the hyphae of *Py. ultimum*. However, there was no *Ps. cepacia* 526 present on *Ph. cinnamomi* hyphae either in the control plates or in any area of the *Ps. cepacia* 526 inoculated plates.

5.3.3. Inhibition of *Ps. cepacia* 526 on agar media by *Ph. cinnamomi* and *Py. ultimum* and by *Ph. cinnamomi* metabolites (exudates).

In these experiments, reductions in the numbers of the bacterial colonies, the sizes of colonies and the average population per colony were used as the measurements of inhibition of *Ps. cepacia* 526 by *Ph. cinnamomi* or by *Py. ultimum*.

Ph. cinnamomi inhibited *Ps. cepacia* 526, reducing the numbers of the bacterium colonies, the sizes of colonies and the average population per colony (Table 5.1). Duncan's multiple range tests showed that *Ph. cinnamomi* had significantly inhibited the bacterium on media containing oatmeal, although the fungus was ineffective on nutrient agar without oatmeal (Table 5.1).

Ph. cinnamomi metabolites (exudates) also inhibited *Ps. cepacia* 526 by reducing the numbers of the bacterium colonies, on media containing oatmeal but not on nutrient agar without oatmeal. However, after a further 5 day incubation, *Ps. cepacia* 526 colonies grew to a similar size regardless of the treatment (Table 5.2). The pH of the agar media as indicated with litmus papers was not altered by the presence of *Ph. cinnamomi*.

Py. ultimum, on the other hand, did not inhibit *Ps. cepacia* 526 on any media tested (Table 5.3). The sizes of the *Ps. cepacia* 526 colonies were not measurable after 5 day further incubation, because the margins of the bacterial colonies became unclear due to the quick bacterial movement towards the *Py. ultimum* hyphae outside the millipore filter paper discs. This was distinctly different from the characteristics of the *Ps. cepacia* 526 colonies in the presence of *Ph. cinnamomi* where the bacterial colonies remained with well defined

margins. This may indicate a chemotaxis of the bacterium towards the *Py. ultimum* hyphae.

5.3.4. The association of nutrients with *Ph. cinnamomi* antibiotic production.

The result (Table 5.4) showed that *Ph. cinnamomi* inhibited *Ps. cepacia* 526 significantly only on the agar medium containing 100 µg thiamine hydrochloride per liter. In terms of colony size and colony density, the growth of *Ph. cinnamomi* was not influenced by thiamine hydrochloride.

5.3.5. Extraction of antibiotic material from *Ph. cinnamomi* liquid culture and the bioassays.

The results of the bioassays are shown in Tables 5.5. and 5.6. None of the fractions including *Ph. cinnamomi* ~~dead~~^{dead} mycelium, the mycelium ethanol extract, the filtered *Ph. cinnamomi* liquid culture, the aqueous fraction of the liquid culture, and the diethyl ether extract of the liquid culture at various concentrations inhibited *Ps. cepacia* 526. The bacterium grew equally well on both nutrient agar and oatmeal agar.

The initial pH of the liquid medium for culturing *Ph. cinnamomi* was 6.57. After *Ph. cinnamomi* was cultured for 7 days, the pH of the liquid culture became 4.45.

5.3.6. Extraction of antibiotics from *Ph. cinnamomi* and *Ps. cepacia* 526 mixed liquid culture and from *Ps. cepacia* 526 liquid culture and their bioassays.

The results of the bioassays are shown in Table 5.7. Duncan's multiple range test showed that neither filtered *Ph. cinnamomi* and *Ps. cepacia* 526 mixed culture, nor the filtered *Ps. cepacia* 526 liquid culture, nor the 100 time concentrated diethyl ether extracts of these two liquid cultures showed any inhibition on *Ps. cepacia* 526. The bacterium again did not show difference in growth when grown on nutrient and oatmeal agars.

Table 5.1. Inhibition of *Ps. cepacia* 526 on agar media by *Ph. cinnamomi*.

Agar media (O*NB)	No. of colonies		Colony diam. (mm)		Population (cfu/colony)	
	Control	<i>Ph. cinnamomi</i>	Control	<i>Ph. cinnamomi</i>	Control	<i>Ph. cinnamomi</i>
0%*0.8%	22 a	21 a	1.05 a	1.05 a	4.9*10 ⁷ a	4.8*10 ⁷ a
1%*0.8%	24 a	14 b	1.02 a	0.27 b	4.8*10 ⁷ a	1.4*10 ⁷ b
4%*0.8%	23 a	12 b	1.05 a	0.24 b	5.0*10 ⁷ a	5.0*10 ⁶ c

O*NB = Oatmeal*Nutrient Broth.

Values are means of four replicate plates. Means of the same nature followed by the same letter are not significantly different according to Duncan's multiple range test at $P < 0.01$; CV = 8.3%.

Table 5.2. Inhibition of *Ps. cepacia* 526 on agar media by *Ph. cinnamomi* metabolites (exudates).

Agar media (O*NB)	Number of colonies		Colony diam. (mm)	
	Control	<i>Ph. cinnamomi</i>	Control	<i>Ph. cinnamomi</i>
0%*0.8%	15a	16a	1.2a	1.2a
1%*0.8%	16a	8b	1.2a	1.15a
4%*0.8%	15a	7b	1.2a	1.2a

O*NB = Oatmeal*Nutrient Broth.

Values are means of four replicate plates. Means of the same nature followed by the same letter are not significantly different according to Duncan's multiple range test at $P < 0.01$; CV = 10.9%.

Table 5.3. Effect of *Py. ultimum* on *Ps. cepacia* 526 on agar media.

Agar media (O*NB)	Number of colonies	
	Control	<i>Py. ultimum</i>
0%*0.8%	22	23
1%*0.8%	24	22
4%*0.8%	23	22

O*NB = Oatmeal*Nutrient Broth.

Values are means of four replicate plates.

Table 5.4. Inhibition of *Ps. cepacia* 526 on agar media by *Ph. cinnamomi*.

Agar media	Number of colonies		Mean size of single colony (mm)	
	Control	<i>Ph. cinnamomi</i>	Control	<i>Ph. cinnamomi</i>
NB	19a	16a	1.56a	1.56a
NB*G	20a	17a	1.53a	1.55a
NB*KNO ₃	18a	19a	1.55a	1.55a
NB*G*KNO ₃	7a	17a	1.48a	1.53a
NB*G*KNO ₃ *T	20a	8b	1.54a	0.35b

NB =Nutrient Broth; G=Glucose; T=Thiamine hydrochloride.

Values are means of four replicate plates. Means of the same nature followed by the same letter are not significantly different according to Duncan's multiple range test at $P < 0.01$; CV = 6.9%.

Table 5.5. Effect of *Ph. cinnamomi* fractions on *Ps. cepacia* 526 on agar media.

Treatment	Measurements on NA		Measurements on OMA	
	No. of colonies	Colony diam. (mm)	No. of colonies	Colony diam.(mm)
Control	21	1.23	22	1.21
Ether control	21	1.21	19	1.21
Filtered liquid culture	18	1.25	20	1.19
Aqueous fraction	23	1.19	18	1.26
Ether extract	22	1.23	20	1.18
Ether extract (10 times concentrated)	24	1.18	19	1.30
Ether extract (100 times concentrated)	22	1.26	24	1.18

Table 5.6. Effect of *Ph. cinnamomi* fractions on *Ps. cepacia* 526 on agar media.

Treatment	Measurements on NA		Measurements on OMA	
	No. of colonies	Colony diam. (mm)	No. of colonies	Colony diam.(mm)
Control	15	1.23	14	1.30
Ethanol control	11	1.25	14	1.22
Dead mycelium	12	1.19	12	1.23
Mycelium ethanol extract	12	1.20	13	1.26

Table 5.7. Effects of the *Ph. cinnamomi* and *Ps. cepacia* 526 mix culture and its extract on *Ps. cepacia* 526 on agar media.

Treatment	Measurements on NA		Measurements on OMA	
	No. of colonies	Colony diam. (mm)	No. of colonies	Colony diam.(mm)
Control	13	0.98	11	1.04
Ether control	10	1.12	10	1.00
Filtered liquid culture of <i>Ph. cinnamomi</i> & <i>Ps. cepacia</i> 526	12	1.05	9	1.00
Filtered liquid culture of <i>Ps. cepacia</i> 526	10	0.97	9	1.13
Ether extract of mixed culture of <i>Ph. cinnamomi</i> & <i>Ps. cepacia</i> 526	11	1.06	10	0.99
Ether extract of culture of <i>Ps. cepacia</i> 526	11	1.08	11	1.04

Table 5.8. Inhibition of *Ps. cepacia* 526 by *Ph. cinnamomi* in liquid media.

Agar media (O*NB)	Control	<i>Ph. cinnamomi</i>
0%*0.8% (pH6.9)	4.98*10 ⁹ a (pH7.4)	4.25*10 ⁹ a (pH7.3)
1%*0.8% (pH6.9)	4.59*10 ⁹ a (pH7.5)	4.35*10 ⁹ a (pH7.4)
2%*0.8% (pH6.8)	4.25*10 ⁹ a (pH7.5)	1.40*10 ⁹ b (pH4.8)

O*NB = Oatmeal*Nutrient Broth.

Values are means of three replicate flasks (cfu/ml). Means followed by the same letter are not significantly different according to Duncan's multiple range test at $P < 0.01$; CV = 14.4%.

5.3.7. The inhibition of *Ps. cepacia* 526 in liquid culture by *Ph. cinnamomi*.

After 7 days incubation, the *Ps. cepacia* 526 populations in both *Ph. cinnamomi* and *Ps. cepacia* 526 mixed culture and in *Ps. cepacia* 526 culture in different media were measured by serial dilution and plating out on nutrient agar. It was found by Duncan's multiple range test that *Ps. cepacia* 526 was significantly ($P=0.05$) inhibited by *Ph. cinnamomi* in liquid medium containing 0.8% nutrient broth and 2% oatmeal, but not in media containing 0.8% nutrient broth or 0.8% nutrient broth plus 1% oatmeal (Table 5.8).

The initial pH values of the different liquid media and that of the liquid cultures were measured (Table 5.8).

5.4. Discussion and Conclusion.

The results presented in chapter 3 showed that the interaction in soil between *Py. ultimum* and *Ps. cepacia* 526 was different from that between *Ph. cinnamomi* and the bacterium. This was confirmed by results from the culture experiments (Chapter 4) which also showed that the inhibition by *Ps. cepacia* 526 of *Ph. cinnamomi* was influenced by the nutrient composition whereas that of *Py. ultimum* was not. It was therefore likely that *Ph. cinnamomi* and *Py. ultimum* may vary in their reactions to *Ps. cepacia* 526 antagonism⁴. It was also indicated that *Ps. cepacia* 526 produced different antibiotics differentially active against *Py. ultimum* and *Ph. cinnamomi*. Experiments in this chapter were thus concentrated on the reactions of *Ph. cinnamomi* and *Py. ultimum* to *Ps. cepacia* 526. The effects of pyrrolnitrin, an antibiotic produced by *Ps. cepacia* 526, on *Ph. cinnamomi* and *Py. ultimum* were also studied.

Pyrrolnitrin is stable in agar media for several days (Mr R. Rickards, personal communications) but, in the plate assay, did not inhibit the growth of either fungus. Thus, pyrrolnitrin was probably not responsible for the

antagonistic effects of *Ps. cepacia* 526 reported in chapter 4. The results also cast doubt upon the role of pyrrolnitrin in the biological control by *Ps. cepacia* 526 of turnip damping-off caused by *Py. ultimum* in soil (Chapter 3). The ineffectiveness of pyrrolnitrin against *Py. ultimum* agrees with a previous report (Howell & Stipanovic 1979).

~~Except for~~ ^{In addition to} pyrrolnitrin, *Ps. cepacia* 526 is also known to produce aminopyrrolnitrin and xylocandin (Hebbar 1986, Dr P.J. Dart & Mr R. Rickards, personal communications). Unfortunately, neither aminopyrrolnitrin nor xylocandin was available for test. Whether *Ps. cepacia* 526 produced more than one antibiotic differentially inhibitory against *Py. ultimum* and *Ph. cinnamomi* is not finally resolved.

Experiments were then concentrated on the effects of *Ph. cinnamomi* and *Py. ultimum* on the bacterium. It was found that *Ps. cepacia* 526 quickly moved along the hyphae of *Py. ultimum* but not those of *Ph. cinnamomi*. An explanation, that the hyphal surface of *Py. ultimum* was physically suitable for the movement and reproduction of *Ps. cepacia* 526 but that of *Ph. cinnamomi* was not, was considered unlikely because of the taxonomic similarity between *Ph. cinnamomi* and *Py. ultimum*. It was more likely that *Ps. cepacia* 526 was chemically inhibited by *Ph. cinnamomi*, but not by *Py. ultimum*. A stimulatory effect on *Ps. cepacia* 526 by *Py. ultimum* may have existed.

Experiments showed inhibition of *Ps. cepacia* 526 by *Ph. cinnamomi* and its metabolites on agar media containing oatmeal, but no inhibition occurred on agar media without oatmeal. As there were no changes in the pH of agar media, *Ph. cinnamomi* did not inhibit the bacterium growth by changing the medium pH. Inhibition of *Ps. cepacia* 526 by the production of antibiotics by *Ph. cinnamomi* therefore remains likely. That *Ph. cinnamomi* metabolites

(exudates) failed to inhibit *Ps. cepacia* 526 after 5 days further incubation suggests that the antibiotics were short-lived.

Oatmeal has consistently shown an effect on the interactions between *Ph. cinnamomi* and *Ps. cepacia* 526 (Chapter 4). In the experiments of this chapter, the presence of oatmeal resulted in the inhibition of *Ps. cepacia* 526 by *Ph. cinnamomi*, suggesting that the differences in nutrient compositions between oatmeal and nutrient broth affected the antibiotic production by *Ph. cinnamomi*.

Although the precise nutrient components of oatmeal are not known, it contains more carbohydrates than does nutrient broth, which is of animal origin and consists of beef extract and Bacto-peptone (Difco Laboratories 1971). The higher carbohydrate content was considered to be possibly significant. An experiment conducted to study the effects of various nutrient components on *Ph. cinnamomi* antibiotic production, however, revealed that thiamine was the essential component, virtually absent in nutrient broth (Difco Laboratories 1971).

Py. ultimum caused no inhibition of the growth of *Ps. cepacia* 526 on any media tested. On the other hand, *Ps. cepacia* 526 tended to move toward the *Py. ultimum* colonies outside the millipore filter paper discs. This may have indicated a chemical attraction from the fungus.

Dr G.A. Chilvers (personal communication) suggested that the effect of oatmeal might be attributed to a thicker mycelial mat between the media and the millipore filter paper providing a barrier to bacteria. This can, however, be excluded for three reasons. The first is that the 3 day old fungal colonies were equally sparse on both medium types when the bacterium was inoculated, although the fungal colonies on oatmeal nutrient broth agar did become denser after *Ph. cinnamomi* grew over the whole plate, normally after 7 days. The second is that *Ph. cinnamomi* colonies were equally dense on media with or without thiamine yet the inhibition occurred only in thiamine-amended

medium. The third is that *Py. ultimum* grew much more densely than *Ph. cinnamomi* on any media used yet did not inhibit *Ps. cepacia* 526.

Attempts to isolate antibiotics from *Ph. cinnamomi* liquid cultures disappointingly failed. Neither ~~*Ph. cinnamomi*~~ ^{dead} mycelium, nor the mycelium ethanol extract, nor the filtered *Ph. cinnamomi* liquid culture, nor the aqueous fraction of the liquid culture, nor diethyl ether extract of the liquid culture at various concentrations showed any inhibition of *Ps. cepacia* 526. The same results obtained from experiment to isolate antibiotics from *Ph. cinnamomi* and *Ps. cepacia* 526 mixed liquid cultures excluded the possibility that *Ps. cepacia* 526 was needed to induce *Ph. cinnamomi* antibiotic production. The population of *Ps. cepacia* 526 was, however, reduced by *Ph. cinnamomi* in the liquid culture containing 2% oatmeal.

The mechanisms by which *Ph. cinnamomi* inhibited *Ps. cepacia* 526 may have been different on agar media and in liquid media. For example, for *Ph. cinnamomi* to effectively inhibit *Ps. cepacia* 526, 2% oatmeal was required for the liquid media but only 1% for agar media in experiments reported in this chapter and in chapter 4.

It seems evident that the inhibition on agar media was antibiotic related and that antibiotic production by *Ph. cinnamomi* was related to the presence of thiamine. There was no pH change by *Ph. cinnamomi* on agar media so *Ps. cepacia* 526 was unlikely to have been inhibited through media pH change. Furthermore, any pH hypothesis does not accord with the evidence on the role of thiamine.

The liquid culture experiments remain to be discussed. There could be many explanations for the population reduction of *Ps. cepacia* 526 by *Ph. cinnamomi* in the liquid cultures. Firstly, *Ph. cinnamomi* might be able to grow efficiently at high nutrient levels even when accompanied by the bacterium. This

was supported by the results in chapter 4 showing that *Ph. cinnamomi* was able to overcome the antibiotic effect of *Ps. cepacia* 526 cell free filtrate to grow efficiently in liquid media of high nutrient concentrations. Secondly, the pH changes in the liquid media may have played roles in the population reduction of *Ps. cepacia* 526. Any effect of changes in pH of the liquid medium caused by the growth of the fungus, however, is unlikely to be of primary significance because the fungus would need to have grown significantly in the presence of the bacterium before pH change could have been induced. Thirdly, *Ph. cinnamomi* may have produced short-lived antibiotics in liquid medium as it did on agar media. A combination of the first and the second explanations or the first and the third may approximate the truth .

**CHAPTER 6. THE GROWTH AND REPRODUCTION OF *PHYTOPHTHORA*
CINNAMOMI AND *PYTHIUM ULTIMUM* AND THE EFFECTS
OF *PSEUDOMONAS CEPACIA* 526**

6.1. Introduction.

The results in chapter 3 showed that *Ph. cinnamomi* caused turnip damping-off and reduced the plant weight only at 0 kPa, and reduced the plant root weight at soil matric potentials between 0 and -5.0 kPa in the high temperature glasshouse, but the same fungus in the constant lower temperature room caused damping-off, reduced the plant weight and the plant root weight at all soil matric potentials tested. A direct association between the disease incidence and the soil temperature and matric potentials was therefore established. One aspect of the importance of soil temperature and matric potentials in the disease incidence relates to the plant predisposition, already discussed in chapter 3. Alternatively, the soil temperatures and matric potentials may have directly affected the pathogen. The high temperatures of soils at the lower matric potentials in the glasshouse may have inactivated the pathogen and consequently reduced the disease, whereas the suitable temperatures in the room favoured the pathogen and consequently increased the disease.

Both mycelium and zoospores of *Ph. cinnamomi* may be involved in infection of plants (Marx & Bryan 1969, Malajczuk & Theodorou 1979). It is likely that the soil temperature and the matric potential affected the disease incidence through their direct influences on *Ph. cinnamomi* in either its mycelial growth, or its sporangial production and zoospore release, or both, in the glasshouse and in the constant temperature room. This chapter reports results of experiments

which examined the sporangial production and zoospore release by *Ph. cinnamomi* in soils at various soil matric potentials.

A plant fungal pathogen usually produces several morphologically and functionally different stages in its life cycle. A potential ~~plant-disease~~ biological control microorganism may significantly affect the growth, production and conversion of spores, saprophytism and survival of the pathogen, and consequently in the diseases it causes. Disease control by such a microorganism may have resulted from its antagonism either to a specific stage or to the whole life cycle of the pathogen. Understanding such effects by the microorganism is therefore important for its successful application and, in the case of failure of controlling the disease, for the explanation of its ineffectiveness and for the improvement of further application.

In chapter 3, it was shown that *Pseudomonas cepacia* 526 effectively reduced damping-off caused by *Pythium ultimum*, but ~~that~~ ^{not} by *Phytophthora cinnamomi* in soil. Experiments reported in chapters 4 & 5 showed that *Ph. cinnamomi* was able to produce antibiotics which inhibited the growth of *Ps. cepacia* 526, and therefore suggested why *Ps. cepacia* 526 was effective in the biological control of disease caused by *Py. ultimum* but ineffective in controlling disease caused by *Ph. cinnamomi*. However, such evidence is not conclusive due to the differences between the artificial culture and soil conditions. More evidences ^{is} ~~are~~ thus desirable. This chapter reports experiments conducted *in vitro* and in soil to examine further the direct effects of *Ps. cepacia* 526 on *Ph. cinnamomi* mycelial growth, sporangial production and zoospore release, and on *Py. ultimum* mycelial growth.

Ph. cinnamomi produces four spore stages including sporangia, zoospore, chlamydospores and oospores in soil (Zentmyer 1980), but it does not normally produce sporangia and release zoospores on agar media (Manning & Crossan

1966, Chen & Zentmyer 1970). However, a method to produce sporangia and zoospores in axenic culture has been developed (Chen & Zentmyer 1970). *Ph. cinnamomi* mycelial mats are washed with a sterile mineral salt solution amended with chelated iron solution so that large number of sporangia are obtained. The sporangia-bearing mycelial mats are chilled at 4°C for 20 min and then returned to 24-26°C. Sporangia release zoospores within 1 h after chilling. This method has been routinely used for the genetic studies of *Ph. cinnamomi* in the Research School of Biological Science, ANU. I have also successfully obtained large number of sporangia and zoospores by this method using the *Ph. cinnamomi* isolate studied throughout this thesis.

The method is often modified and used to study the effects of soil microorganisms on the sporangial production and zoospore release by *Ph. cinnamomi*. The modified method uses soil leachates or suspension of soil microorganism, instead of the sterile mineral salt solution amended with chelated iron solution.

Bacteria in soil leachates were found to be associated with formation and breakdown of sporangia in *Phytophthora* spp., including *Ph. cinnamomi* (Ayers & Zentmyer 1971, Broadbent & Baker 1974, Chee & Newhook 1966, Manning & Grossan 1966, Zentmyer 1965). Culture filtrates of the mycorrhizal fungus *Leucopaxillus cerealis* var. *piceina* slowed the growth of *Ph. cinnamomi* and completely inhibited zoospore germination (Marx 1969). Suppression of *Ph. cinnamomi* in soil has been shown to be associated with soil microorganisms which accumulated on the fungal propagules and resulted in hyphal lysis, sporangium abortion and reduced germination (Malajczuk *et al.* 1977, Malajczuk 1979, Malajczuk & Theodorou 1979, Malajczuk 1988, Murray 1987). In the experiments of this chapter, the modified method was used, utilizing both

artificial media and non-sterile soil so that the results can be compared and the conclusion made more convincing.

Py. ultimum produces sporangia, chlamydospores and oospores, but rarely zoospores (Drechsler 1946, Hendrix & Campbell 1973). Mycelium and sporangia are important in plant infection, with the latter usually produces germ tubes, and both are affected by soil microorganisms (Howell 1981, Howell 1982, Howell & Stipanovic 1980, Howell & Stipanovic 1983, Lifshitz & Hancock 1983, Lifshitz & Hancock 1984, Nelson *et al.* 1986). However, since *Py. ultimum* commonly produces sporangia on agar media and in soil, *Ps. cepacia* 526 was not tested for its effect on the sporangial production of *Py. ultimum*, due to the unavailability of sporangium-free mycelial mats.

6.2. Materials and Methods.

6.2.1. Effects of *Ps. cepacia* 526 and soil leachate on sporangial production and zoospore release by *Ph. cinnamomi* in artificial media.

V-8 agar medium was used for the culture of *Ph. cinnamomi*. A piece of polycarbonate membrane (4.7 cm diameter, 8 μ m pore size, Nucleopore Corp., Pleasanton, Ca.) was placed on the surface of the V-8 agar plate. A 7 day old fungal plug of *Ph. cinnamomi* was then inoculated onto the center of the polycarbonate membrane. The plates were incubated for 7 days at 26°C to allow the formation of a mycelial mat over the membrane.

A *Ps. cepacia* 526 nutrient suspension of 1.5×10^9 was prepared by the method described in 2.2.2. A *Ps. cepacia* 526 cell free filtrate was obtained by centrifuging the bacterial suspension (5000 g for 30 min at 4°C; Sorvall RC-5B Refrigerated Superspeed Centrifuge) and filtering the supernatant through a 0.2 μ m pore size filter (Millipore Corp., Bedford, MA) under sterile condition. A soil leachate was prepared by placing 10 g soil into 1000 ml distilled water, shaking at

200 rpm for one hour, and filtering the suspension through a Whatman No. 4 filter paper. Sterile soil leachate was prepared by autoclaving the soil leachate at 121°C for 30 min. These suspensions were dispensed into 9 cm diameter petri plates (20 ml each) and examined for their effects on sporangial production and zoospore release by *Ph. cinnamomi*. Treatments included: 1) sterile distilled water (20 ml), 2) sterile soil leachate (20 ml), 3) soil leachate (20 ml), 4) *Ps. cepacia* 526 suspension (18 ml sterile distilled water plus 2 ml *Ps. cepacia* 526 suspension), 5) *Ps. cepacia* 526 filtrate (18 ml sterile distilled water plus 2 ml *Ps. cepacia* 526 filtrate), 6) sterile soil leachate and *Ps. cepacia* 526 suspension (18 ml sterile soil leachate plus 2 ml *Ps. cepacia* 526 suspension), and 7) soil leachate and *Ps. cepacia* 526 suspension (18 ml soil leachate plus 2 ml *Ps. cepacia* 526 suspension).

After *Ph. cinnamomi* was cultured for 7 days, the membranes were peeled from the agar plates, with the hyphae having penetrated the pores and become attached to the surface. One membrane was immediately mounted on a microscope slide and no sporangial production was observed at this stage. The other membranes were placed into petri plates containing 20 ml of the different suspensions listed above, one membrane in each petri plate with three replicates for each suspension. The petri plates were incubated for 8 days at 26°C. The membranes were recovered from the petri plates and mounted on microscope slides. Total numbers of sporangia on each membrane were estimated by random inspections of 10 microscopic fields at x100 magnification.

Immediately after the examination of sporangial production, the membranes were again put back and immersed in the suspensions where they had been treated and chilled at 4°C for 30 min. They were then incubated at 26°C. The membranes were recovered from the petri plates and mounted on microscope slides. The total numbers of sporangia that released zoospores on

each membrane were estimated by random inspections of 10 microscopic fields at x100 magnification after 1.5 h, 6 h, 24 h and 48 h incubation at 26°C.

6.2.2. Sporangial production and zoospore release by *Ph. cinnamomi* and the effects of *Ps. cepacia* 526 at various soil matric potentials.

Soil matric potentials were controlled at 0, -1.5, -3.0, -5.0 and -10.0 kPa in the constant temperature room using the method described in 3.2.2. *Ph. cinnamomi* mycelial mats (7 day old) supported by the polycarbonate membrane were obtained using the method described above in section 6.2.1. These membranes were placed on the surfaces of the soil in the Buchner funnels, with one membrane in each funnel. The membranes were covered with an approximately 1 cm layer of soil. *Ps. cepacia* 526 nutrient broth suspension (5 ml containing 1.2×10^9 cfu/ml) was then sprayed into each Buchner funnel. More water was sprayed into each Buchner funnel to saturate the soil so that the bacteria were drained into the soil uniformly during the quick process of water ~~equilibration~~ ^{equilibrium}. An equal amount of nutrient broth without *Ps. cepacia* 526 was sprayed into each control Buchner funnel. There were three replicates for each treatment.

After 7 days, the membranes were recovered from the Buchner funnels and rinsed in sterile distilled water to remove the soil particles. They were then mounted on microscope slides. Total numbers of sporangia and the numbers of sporangia that released zoospores on each membrane were estimated by random inspections of 10 microscopic fields at x100 magnification.

6.2.3. The mycelial growth of *Ph. cinnamomi* and *Pythium ultimum* and the effects of *Ps. cepacia* 526 at various soil matric potentials.

Soil matric potentials were controlled at 0, -1.5, -3.0 kPa in the constant temperature room. Mycelial mats of 7 day old of both *Ph. cinnamomi* and *Py. ultimum* supported by the polycarbonate membrane were obtained using method

as described above in section 6.2.1. A nutrient suspension containing 1.7×10^9 cfu/ml *Ps. cepacia* 526 was prepared and used in this test. The application of fungal mycelial mats and *Ps. cepacia* 526 nutrient broth suspension and the control used were as described above in section 6.2.2. There were three replicates for each treatment.

After 7 days, the membranes were recovered from the Buchner funnels and rinsed in sterile distilled water to remove the soil particles. The mycelial mats were stained with filtered aniline blue solution (5% aqueous phenol 15 ml : 1% aqueous aniline blue 1 ml : glacial acetic acid 4 ml) for 10 min, and then rapidly washed and dehydrated in 95% ethanol (Jones & Mollison 1948, Nesbitt *et al.* 1979). The mycelial mats were then mounted on microscope slides and examined at x100 magnification with a calibrated cross-hatched grid consisting of 100 intersections. At each intersection, hyphae that were stained blue were determined as living and hyphae that were unstained were determined as lysed. Random inspections of 10 microscopic fields were made for each membrane of the three replicates.

6.3. Results.

6.3.1. The effects of *Ps. cepacia* 526 and soil leachate on sporangial production and zoospore release by *Ph. cinnamomi* in artificial media.

After the polycarbonate membranes were recovered from the petri plates and mounted on microscope slides, the total numbers of sporangia on each membrane were estimated by random inspections of 10 microscopic fields at x100 magnification. *Ph. cinnamomi* was able to form sporangia in all treatments. The results analyzed by analysis of variance showed there were no differences between the treatments, including sterile distilled water (Table 6.1). This indicated that *Ps. cepacia* 526 in this test had no effect on *Ph. cinnamomi*

sporangial production. Chilling the sporangia formed in all treatments at 4°C for 30 min failed to induce any zoospore release.

6.3.2. The sporangial production and zoospore release by *Ph. cinnamomi* and the effects of *Ps. cepacia* 526 at various soil matric potentials.

After the membranes were recovered from the Buchner funnels and rinsed in sterile distilled water to remove the soil particles, they were mounted on microscope slides. The total numbers of sporangia and the numbers of sporangia that released zoospores on each membrane were estimated by random inspections of 10 microscopic fields at x100 magnification. The results was analyzed by analysis of variance. *Ph. cinnamomi* only produced sporangia in saturated soil (Fig. 6.1). Most of the sporangia so produced released zoospores. Sporangial production and zoospore release were not affected by the addition of *Ps. cepacia* 526 in the soil.

6.3.3. The mycelial growth of *Ph. cinnamomi* and *Pythium ultimum* and the effects of *Ps. cepacia* 526 at various soil matric potentials.

The membranes supporting either *Ph. cinnamomi* or *Py. ultimum* mycelial mats were recovered from the Buchner funnels, rinsed in sterile distilled water to remove the soil particles and stained with filtered aniline blue solution. The mycelial mats were then mounted on microscope slides and examined for the hyphal lysis. The results were recorded as percentage of lysis. It was found that hyphal lysis occurred in both *Ph. cinnamomi* and *Py. ultimum* (Figures 6.2 & 6.3) and was not affected by the soil matric potentials tested. The addition of *Ps. cepacia* 526 in soil significantly increased hyphal lysis of *Py. ultimum* (Fig. 6.4) but had no effect on that of *Ph. cinnamomi* (Fig 6.5). There was no interaction between the effects of *Ps. cepacia* 526 and soil matric potentials tested.

Table 6.1 Effects of *Ps. cepacia* 526 and soil leachate on *Ph. cinnamomi* sporangium production.

Treatments	No. of sporangia [#] / 10 microscopic fields	
Sterile distilled water	239	a
Sterile soil leachate	264	a
Soil leachate	276	a
<i>Ps. cepacia</i> 526	256	a
<i>Ps. cepacia</i> 526 filtrate	286	a
Sterile soil leachate+ <i>Ps. cepacia</i> 526	249	a
Soil leachate+ <i>Ps. cepacia</i> 526	282	a

[#]Numbers are means of three replicates

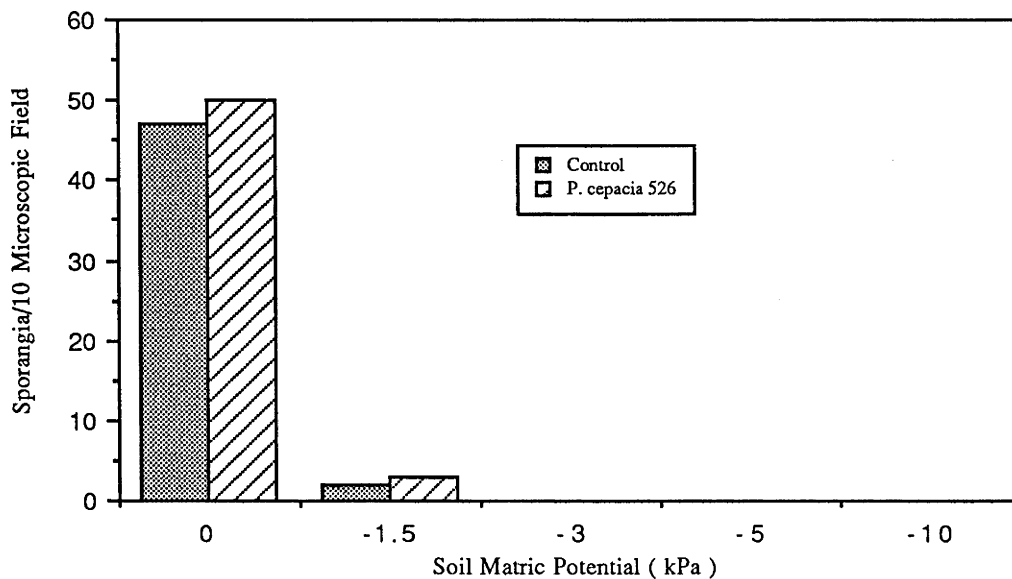
*****ANALYSIS OF VARIANCE*****

Variate: ln (sporangia/10 microscopic fields)

Source of variation	DF	SS	SS%	MS	VR	F
Treatments	6	15.32	36.04	2.56	1.32	
Residual	14	27.19	63.96	1.94		
Total	20	42.51	100	2.13		

SE=0.3610, CV%=13.2

Fig. 6.1. *Ph. cinnamomi* sporangium production and the effect of *Ps. cepacia* 526 at various soil matric potentials



*****ANALYSIS OF VARIANCE*****

Variate: $\ln(\text{sporangia}/10 \text{ microscopic fields})$

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	4	9.32	62.26	2.33	11.10	4.43 (P=0.01)
<i>Ps. cepacia</i>	1	0.45	3.01	0.45	2.14	
Potentials* <i>Ps. cepacia</i>	4	1.01	6.75	0.25	1.19	
Residual	20	4.19	27.98	0.21		
Total	29	14.97	100.00	0.52		

SE=0.1610, CV%=8.5

Fig. 6.2. Hyphal lysis of *Py. ultimum* in soil at -3.0 kPa.

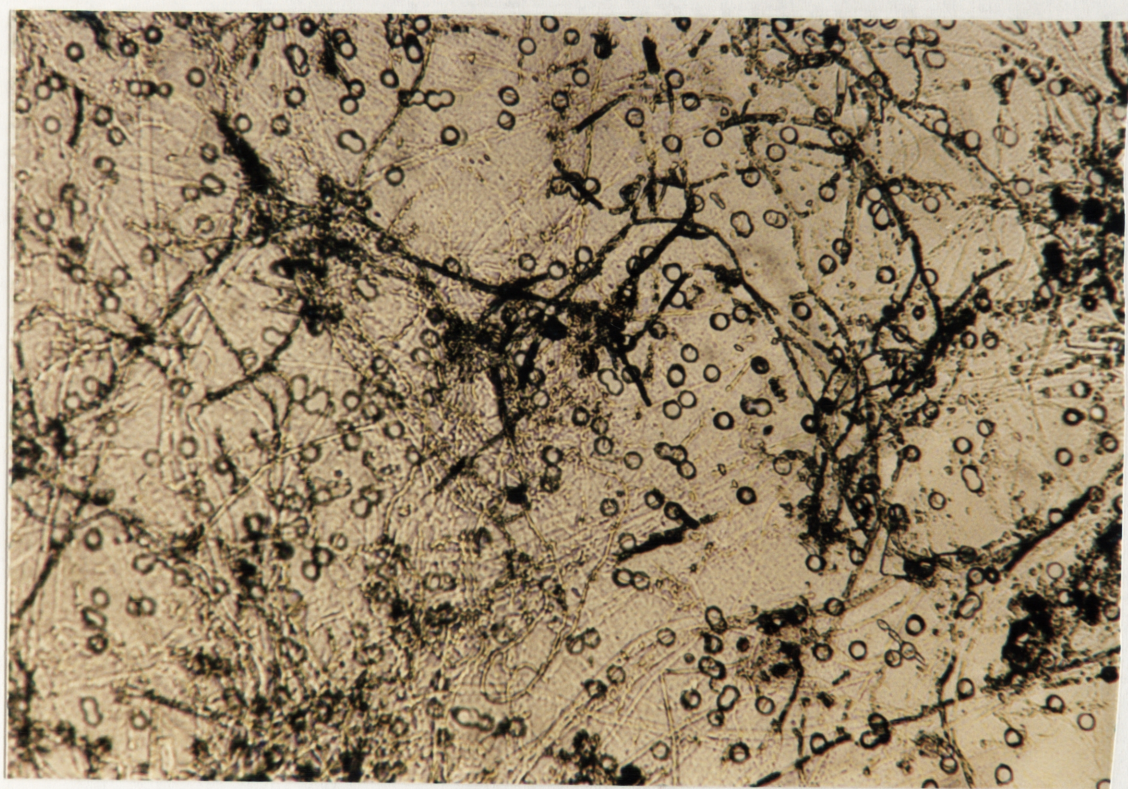
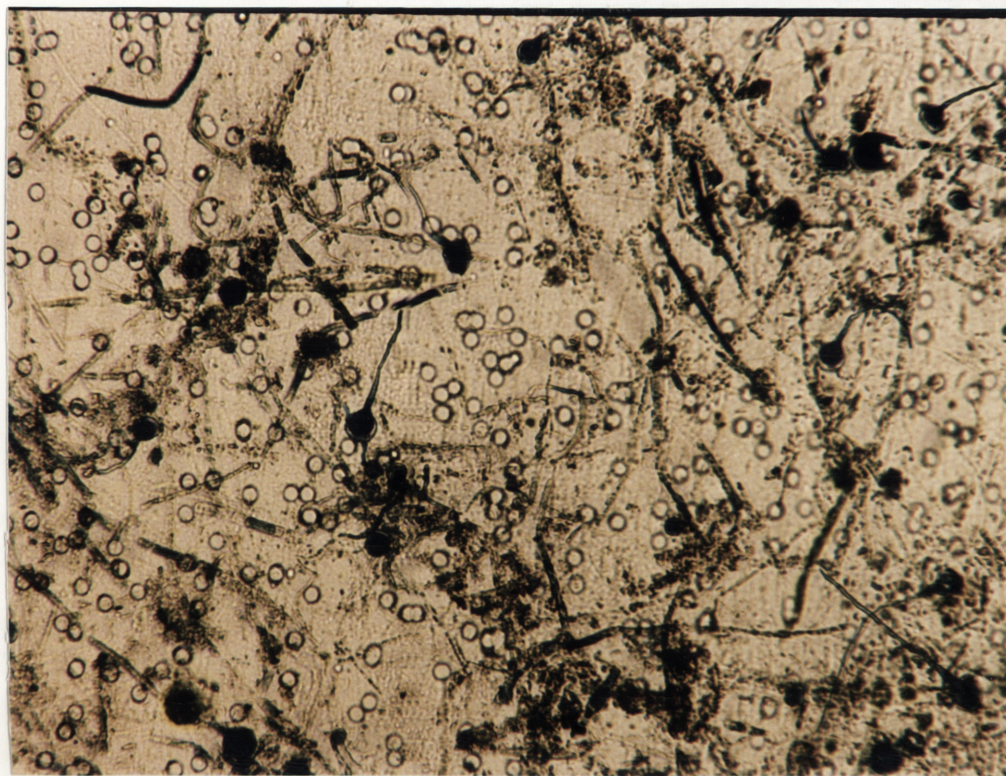


Fig. 6.3. Hyphal lysis of *Ph. cinnamomi* in soil at -3.0 kPa.

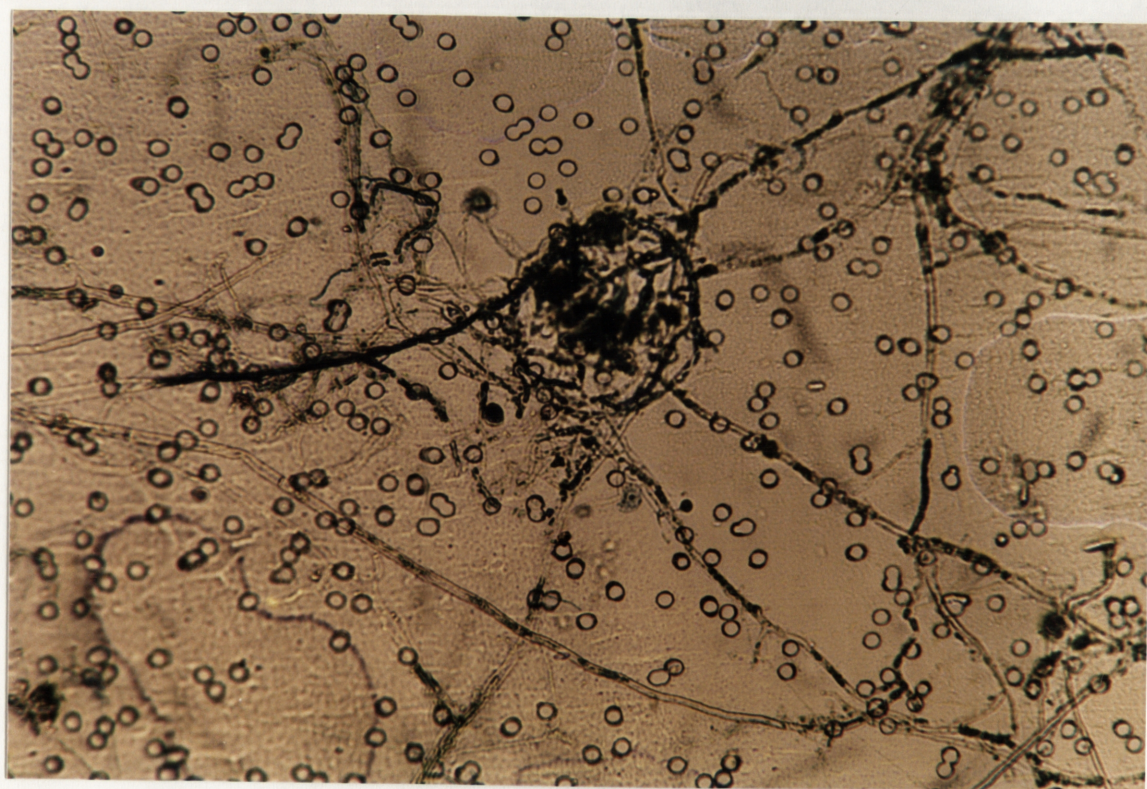
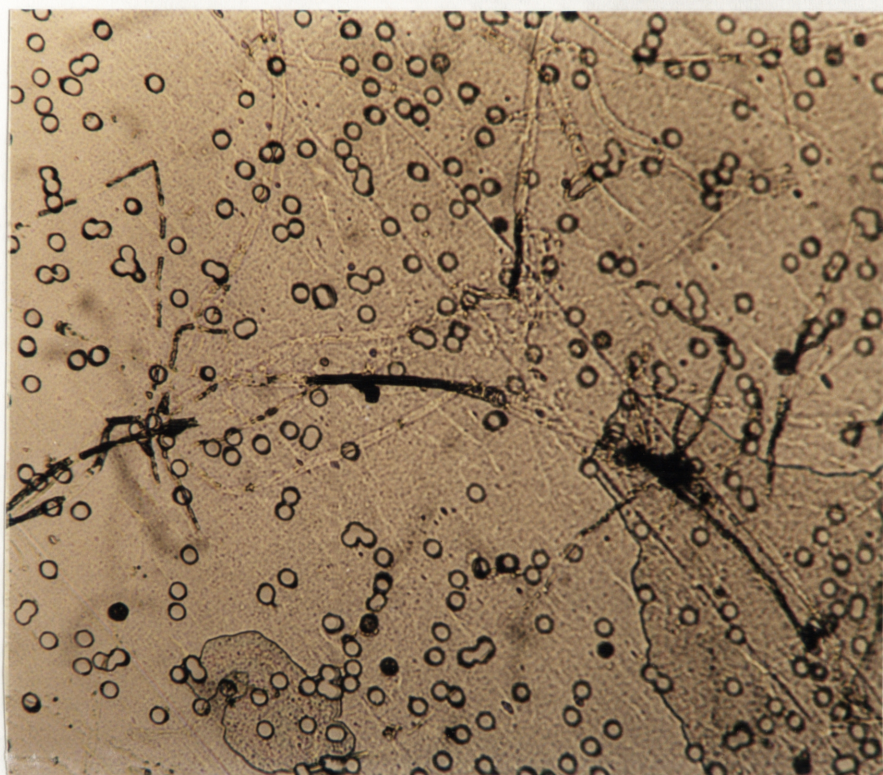
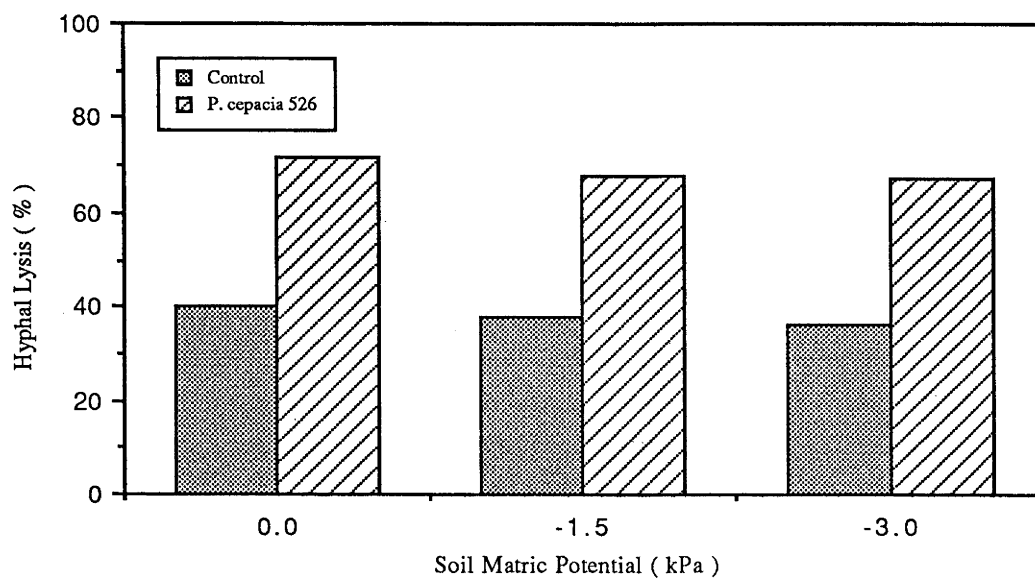


Fig. 6.4. *Py. ultimum* hyphal lysis, with or without *Ps. cepacia* 526, in soil at various matric potentials.



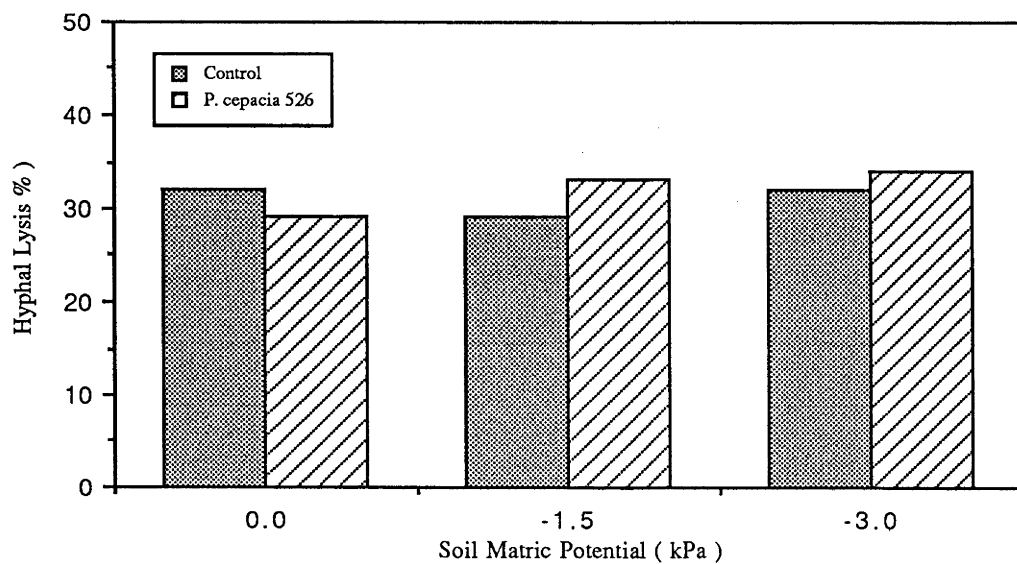
*****ANALYSIS OF VARIANCE*****

Variate: \sin^{-1} (square root of % hyphal lysis)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	2	99.62	14.21	49.81	1.73	
<i>Ps. cepacia</i>	1	189.44	27.02	189.44	6.59	4.75 (P=0.05)
Potentials* <i>Ps. cepacia</i>	2	67.00	9.56	33.50	1.17	
Residual	12	345.00	49.21	28.75		
Total	17	701.06	100.00	41.24		

SE=3.610, CV%=7.3

Fig. 6.5. *Ph. cinnamomi* hyphal lysis, with or without *Ps. cepacia* 526, in soil at various matric potentials.



*****ANALYSIS OF VARIANCE*****

Variate: \sin^{-1} (square root of % hyphal lysis)

Source of variation	DF	SS	SS%	MS	VR	F
Potentials	2	72.64	13.28	36.32	1.50	
<i>Ps. cepacia</i>	1	66.15	12.09	66.15	2.73	
Potentials* <i>Ps. cepacia</i>	2	117.06	21.40	58.53	2.41	
Residual	12	291.24	53.23	24.27		
Total	17	547.09	100.00	32.18		

SE=4.510, CV%=11.5

6.4. Discussion and conclusion.

The methods described in this chapter have often been used by other researchers and many results support the close association of soil microorganisms with sporangial production and zoospore release by *Ph. cinnamomi* (refer to 6.1). Possible mechanisms of such effects have been stated to be (1) that the soil microorganisms enhance *Ph. cinnamomi* sporulation by nutrient depletion and (2) that the soil microorganisms produce stimulatory substance. The nutrient depletion explanation, however, should be applied with caution. It was not supported by any quantitative studies. Even when those non-quantitative experiments ~~was~~ ^{were} conducted *in vitro*, the nutrient levels were hardly comparable to the soil nutrient conditions. For example, Murray (1983) studied the effects of several *Penicillium* species on *Ph. cinnamomi* sporangial production. The experiment was conducted by inoculating 20 ml sterile distilled deionized water in a petri dish concurrently with a *Ph. cinnamomi* mycelial mat and two PDA agar discs bearing *Penicillium* sp. (5 mm diameter). Two controls used a *Ph. cinnamomi* mycelial mat with or without two uninoculated PDA agar discs. It was found that a large number of sporangia was produced on the mycelial mats in the dishes inoculated with *Penicillium* spp. but none on either of the controls. Murray concluded that the effects of *Penicillium* spp. were caused by nutrient depletion, i.e. *Penicillium* spp. utilized the nutrient in the agar. He additionally suggested that the *Penicillium* spp. may have also produced substances that stimulated the formation of sporangia. An explanation based on nutrient depletion is however inappropriate given that no sporangium was formed in the control without PDA agar discs, which in the experiment was the major source of nutrient according to the author.

The *in vitro* test in this chapter showed that *Ph. cinnamomi* was able to produce sporangia in distilled water as well as in the soil leachates with or

without *Ps. cepacia* 526, but the sporangia so produced were aborted. The method used in this chapter was different from the standard method for production of sporangia and zoospores only in that the mineral salt solution was not used. It therefore suggests that the mineral salt effects rather than nutrient depletion effects might have existed but never been noticed in the previously reported interactions between *Ph. cinnamomi* and soil microorganisms. It may also indicate that the sterile mineral salt solution normally used to wash the mycelial mats for production of sporangia may be equally important in the zoospore release as ~~is~~ in the formation of sporangia.

Results obtained from both *in vitro* and soil tests in this chapter showed that *Ps. cepacia* 526 neither affected the sporangial production nor increased the lysis of *Ph. cinnamomi* mycelium. This probably excludes the existence of any significant inhibitory effects by *Ps. cepacia* 526 on the fungus in soil, resulting in its ineffectiveness in biologically controlling turnip damping-off caused by *Ph. cinnamomi* (Chapter 3). The mycelial lysis of *Ph. cinnamomi* commonly occurring in soil at various matric potentials tested in this experiment was probably due to the natural soil microflora.

Ps. cepacia 526 caused a significant increase in lysis of *Py. ultimum* mycelium. It seems reasonable that there is a clear linkage between the direct inhibitory effect on *Py. ultimum* and the effective biological control of the disease caused by the fungus by *Ps. cepacia* 526 in soil reported in chapter 3. Again, the mycelial lysis of *Py. ultimum* commonly occurring in soil at various matric potentials tested, in the absence of *Ps. cepacia* 526, was probably due to the natural soil microflora.

Ph. cinnamomi formed sporangia and released zoospores in the constant temperature room, where the soil temperatures were optimal, only in saturated soil. This suggests that zoospores may play an important role in plant infection

and disease incidence only in saturated soils, whereas mycelium is important in non-saturated soils and probably also in saturated soils.

Since *Ph. cinnamomi* caused turnip damping-off, reduced plant weight and plant root weight at all matric potentials tested in the constant temperature room (Chapter 3), it is suggested that the mycelial growth in soil, the plant infection caused by the mycelium, and the subsequent mycelial growth inside the plant tissues and the disease incidence were not affected by the soil temperature and matric potential at that location. Initial infection at 0 kPa by *Ph. cinnamomi* may be from either zoospores or mycelia or both, but must have originated from mycelium at lower matric potentials.

Ph. cinnamomi caused turnip damping-off and reduced plant weight only at 0 kPa, reduced plant root weight at matric potentials between 0 and -5.0 kPa in the high temperature glasshouse (Chapter 3). One possibility is that zoospores were produced, initiated infection and progressed effectively so as to cause damping-off at 0 kPa, even at the soil temperature reached (32.0°C). Another possibility may have also existed that zoospores and plant predisposition by soil saturation, which has been discussed in chapter 3, caused the disease incidence.

Ph. cinnamomi mycelium may be able to initiate infection and cause damage in the glasshouse, but the extent of infection and the consequent damage were largely affected by the soil temperature. At -10.0 kPa (38.5°C) and -15.0 kPa (39.5°C), the mycelia might have been completely inactivated by the high temperature and no infection occurred, whereas at 0 (32.0 °C), -1.5 (32.5°C), -3.0 (37°C) and -5.0 kPa, the mycelia have not been completely inactivated and some infection occurred and damage caused resulting in plant root weight reduction. The plant root weight was probably the most sensitive indication of *Ph. cinnamomi* infection in the study.

The highest temperature of soil at -1.5 kPa (32.5°C) was comparable to that at 0 kPa (32.0°C) in the glasshouse, yet disease and plant weight reduction only occurred at 0 kPa (Chapter 3). Since there was no difference in *Ph. cinnamomi* hyphal lysis between at 0 kPa and at -1.5 kPa, *Ph. cinnamomi* mycelia alone could not possibly be responsible for the disease and plant root weight reduction. However, a possibility still cannot be excluded that *Ph. cinnamomi* mycelium may have infected plant more effectively when the plant was predisposed by soil saturation.

CHAPTER 7. COLONIZATION ABILITY AND GROWTH-PROMOTING EFFECTS OF *PSEUDOMONAS CEPACIA*

7.1. Introduction.

The successful application of a microorganism as a plant disease biological control agent will depend on its fate in soil after being applied. A large population in soil is believed to be a prerequisite for effective action. The population is determined not only by the soil physical and chemical conditions, and by other soil microorganisms, but also by the plant. The rhizosphere effects resulting from plant root exudation have long been recognized (Rovira 1965, Rovira 1965, Rovira & Davey 1974). The ability for a microorganism to utilize efficiently plant root exudates is therefore an important component of success in colonizing the root. In turn, the biological control agent may benefit the host plant by its direct growth-promoting effects, as well as its disease control effects.

There are a few reports of *Ps. cepacia* as a biological control agent (refer to 1.3.3), but information on its ability to colonize the rhizosphere soil and plant roots ~~are~~ ^{is} rare. Hebbar (1986) found *Ps. cepacia* strains to be good colonizers of maize roots, even when the maize seeds were coated in suspensions with an initial inoculum population as low as 10^1 cfu/ml.

Information concerning the ability of *Ps. cepacia* to promote plant growth is also inadequate. The growth-promoting effects of *Ps. cepacia* may be affected by the soil environment conditions but no evidence is available. Cavileer and Peterson (1985) found that *Ps. cepacia* significantly increased plant fresh weight of China aster in the presence of *Fusarium* in a field test.

The results in chapter 3 showed that *Pseudomonas cepacia* 526 had a stimulatory effect on turnip growth by increasing the seedling root weight during the 2 week long experiments, although it did not increase the whole plant

weight. This indicated that *Ps. cepacia* 526 was acting as a plant growth promoting rhizobacterium as well as a plant disease biocontrol agent. Experiments reported in this chapter aimed to study further the colonization by *Ps. cepacia* 526 of turnip root and its effects on turnip seedling growth. At the early stage of the research programme, an experiment was conducted to study the colonization by three *Pseudomonas cepacia* strains of *Pinus radiata* roots and the rhizosphere soil. The results are also reported in this chapter.

7.2. Materials and Methods.

7.2.1. Colonization by *Ps. cepacia* of *Pinus radiata* root and the rhizosphere soil.

7.2.1.1. *Ps. cepacia*, plant and soil.

Three strains of *Ps. cepacia* (56, 406, 526) were tested. *Ps. cepacia* 526 nutrient broth suspension containing 2.5×10^9 cfu/ml was prepared as described in 2.2.2. Nutrient broth suspensions of *Ps. cepacia* 56 (1.4×10^9 cfu/ml) and *Ps. cepacia* 406 (1.3×10^9 cfu/ml) were also prepared in the same way. These suspensions were serially diluted to obtain their 10^7 , 10^5 , 10^3 , 10^1 fold suspensions.

Seeds of *Pinus radiata* (Table 2.1) were surface sterilized by rinsing them in 75% alcohol for 1 min, sterilizing with 1% sodium hypochlorite for 10 min while shaking at 200 rpm, and then rinsing 10 times with sterile distilled water.

Both unsterilized and sterilized soils were used (see 2.4 for details). When sterilized soil was required, the soil was steamed at 121°C , 30 min each day for two consecutive days.

7.2.1.2. Inoculation of seed with bacteria.

Surface-sterilized seeds of *P. radiata* were put into the serially diluted bacterial suspensions and shaken at 200 rpm for one hour. The control used *P. radiata* seeds rinsed and shaken in nutrient broth at 200 rpm for one hour.

The initial *Ps. cepacia* populations on seed were counted. Five seeds inoculated with the same bacterial suspension were randomly sampled and shaken in 100 ml of sterile distilled water. The bacterial suspensions obtained were a 10^2 fold dilution. There were three replicates for seeds from the same bacterial suspension. Serial dilutions were then made by transferring 1 ml into 9 ml sterile distilled water. The samples of these serial dilutions (0.01 ml) were spotted onto a nutrient agar plate, with 3 to 4 dilution levels per plate and three replicate spots per dilution. The agar plates were incubated at 30°C for 48 h to 72 h and the colonies counted. The bacterial counts were converted to numbers of bacterium per seed.

7.2.1.3. Growth of *P. radiata* seedlings.

Either sterilized or unsterilized soil (40 g) was placed into a large glass tube (2.1 cm diameter, 20 cm long) which was blocked at one end with a rubber stopper. 15 ml sterile water was added into each test tube. The glass tubes were held at room temperature for 24 h for the water distribution in the soil to become uniform. This was proven suitable for the growth of *P. radiata* seedlings in a pre-experiment test. One seed of *P. radiata* that had been rinsed in either bacterial suspension or nutrient broth was then sown into the glass tube and covered with 2 g of either sterilized or unsterilized soil. To conserve soil moisture, the top soil in the glass tube was mulched with black nylon chips, and the periphery of the glass tube was wrapped with aluminium foil to reduce heating of the soil. Fifteen replicates were made of each soil-*Ps. cepacia* suspension variable. The glass tubes were incubated in a glasshouse for the growth of seedlings. The glasshouse daily temperatures ranged from 18-36°C.

7.2.1.4. Recoveries of *Ps. cepacia* from plant root and rhizosphere soil.

Four weeks after seedling emergence, the seedlings together with the soil were pushed out from the glass tubes through the end blocked by the rubber

stopper. The seedlings were harvested carefully without damaging their root system. They were used for recovery of *Ps. cepacia* from both the root and the rhizosphere soil.

To obtain the rhizosphere soil, soil loosely and closely attached to the root was washed off by dipping and shaking in 10 ml sterile distilled water. For the recovery from the root, a single root was macerated in 10 ml sterile distilled water with a mortar and pestle.

When the rhizosphere soil had been washed off in 10 ml of sterile distilled water and when the root was macerated in 10 ml of sterile distilled water, the suspensions obtained were a 10^1 fold dilution. Serial dilutions were then made for the rhizosphere soil and the root macerates by transferring 1 ml into 9 ml sterile distilled water. Samples of these serial dilutions (0.01 ml) were spotted onto plates containing rifampicin nutrient agar which is selective to *Ps. cepacia*, with 3 to 4 dilution levels per plate and three replicate spots per dilution. The agar plates were incubated at 30°C for 48 h to 72 h. The rhizosphere soil or the root macerates in the initial 10^1 fold suspension were dried at 75°C and weighed. The actual dry weight of the rhizosphere soil or the root macerates were obtained by the following adjustment:

$$\text{Actual dry weight} = \text{weight in the initial } 10^1 \text{ fold suspension} \times 10/9$$

After plate incubation, *Ps. cepacia* 526 colonies were counted. All the counts were converted to numbers per gram of rhizosphere soil or per gram of root: *Ps. cepacia* population (cfu/g) = *Ps. cepacia* 526 counts/actual weight.

All data for bacterial populations were subjected to analysis of variance and least significant difference test. A natural logarithm-transformation [$\ln(x)$] was applied to the data before statistical analysis was performed.

7.2.2. Colonization and growth-promoting effects of *Ps. cepacia* 526 on turnip.

7.2.2.1. Growth of turnip and application of *Ps. cepacia* 526

A *Ps. cepacia* 526 suspension containing 1.5×10^9 cfu/ml was prepared as described in 2.2.2. Turnip seed and the unsterilized soil were used for this test.

This test was done in the constant temperature room described in chapter 3. Since it was found that the biological activities of *Ps. cepacia* 526 were not affected by the soil matric potentials tested in chapter 3, this test was done at only one matric potential level. The soil matric potential was controlled at -3.0 kPa using the method as described in 2.4.3.

Soil (50 g) was added to a Buchner funnel, wetted and allowed to equilibrate for 24 h. Turnip seeds (30) were sown into the Buchner funnel and covered with a thin layer of soil. *Ps. cepacia* 526 nutrient broth suspension (5 ml) was then sprayed evenly into each Buchner funnel. A small amount of water was sprayed into each Buchner funnel to saturate the soil so that the bacterium was drained into the soil uniformly during the quick process of water ~~equilibrium~~ ^{equilibration}. The control used 5 ml nutrient broth without *Ps. cepacia* 526.

7.2.2.2. Harvest of turnip seedlings

After seeds were sown, seedlings were harvested from three randomly chosen *Ps. cepacia* 526 inoculated Buchner funnels and three control Buchner funnels each week for six consecutive weeks. The seedlings together with soil were pushed out from each Buchner funnel by water pressure from the other side of the tension plate. The seedlings were rinsed in tap water to remove the soil attached to the root. These seedlings from each Buchner funnel were then used for the recovery of *Ps. cepacia* 526 from the root and the measurement of plant weight and plant root weight.

7.2.2.3. Examination of bacterial populations on roots of turnip seedlings and measurements of the plant weight and the plant root weight.

The seedlings were cut into two parts (root and shoot), the plant shoots from the same Buchner funnel were dried together to a constant weight at 75°C and weighed.

For the recovery of bacteria from the root, roots collected from the same Buchner funnel were macerated together in 10 ml of sterile water with a pestle and mortar. The suspension obtained was a 10^1 fold dilution. Serial dilution were made for the root macerates by transferring 1 ml into 9 ml sterile distilled water. The samples (0.1 ml each) of each dilutions were pipetted onto both nutrient agar plates and rifampicin nutrient agar plate and spread with a sterile glass rod. The nutrient agar is generally used for bacterial culture whereas the rifampicin nutrient agar is a selective medium for *Ps. cepacia*. The agar plates were then incubated at 30°C for 48 to 72 h.

The weight of the root macerates in the initial 10^1 fold suspension was obtained by drying it at 75°C and weighing. The weight was again adjusted to obtain the actual dry weight of the root macerates.

After plate incubation, the numbers of bacterial colonies were counted. The counts from the nutrient agar plates were regarded as total bacterial populations, whereas that from the rifampicin nutrient agar plates were regarded as *Ps. cepacia* 526 populations. These counts were converted to numbers per gram of root: bacterial population on root (cfu/g) = bacterial counts/actual root macerates weight.

It was found that the turnip seedling root weight was so small in contrast to the plant shoot weight that a major change in the root weight had little influence on the whole plant weight. In other words, the plant shoot weight was

nearly equal to the whole plant weight. So, the plant weight and the plant root weight were used as measurements of the plant growth.

The plant root weight were obtained by dividing the actual root macerates weight by the number of plant roots.

The plant weight were obtained by dividing the sum of plant shoot weight and root macerate weight by the number of plants.

All data for bacterial populations, plant weight and plant root weight were subjected to least significance test. A natural logarithm-transformation [$\ln(x)$] was applied to the data before statistical analysis was performed.

7.3. Results.

7.3.1. Colonization of *P. radiata* roots and the rhizosphere soil by *Ps. cepacia* .

After seeds of *P. radiata* were put into the serially diluted bacterial suspensions and shaken at 200 rpm for one hour, recoveries of *Ps. cepacia* were made from the inoculated seeds. The results are shown in Fig. 7.1. Recovery of each *Ps. cepacia* strain was proportional to the inoculum population used for seed inoculation. However, the seed population of *Ps. cepacia* 526 was smaller than *Ps. cepacia* 56 and *Ps. cepacia* 406, although the initial concentration of suspensions were higher.

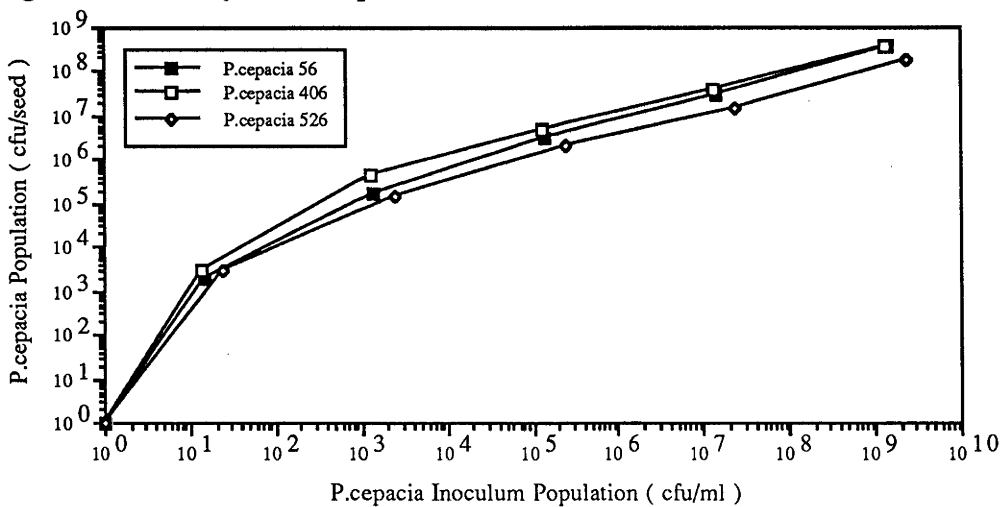
Figures 7.2-4 show the recoveries of *Ps. cepacia* from the rhizosphere soil and the roots of *P. radiata* seedlings grown in both sterilized and unsterilized soils.

In the sterilized soil, all the inoculated strains of *Ps. cepacia* were recovered from the root and the rhizosphere soil of *P. radiata* seedlings, even if the populations of the inocula used for seed inoculation were as low as 10^1 cfu/ml. Significant differences existed in the populations on the root and in the rhizosphere soil, resulting from the different populations of the inocula used.

The higher the inoculum population, the greater the population recovered. No *Ps. cepacia* was recovered from the control plants.

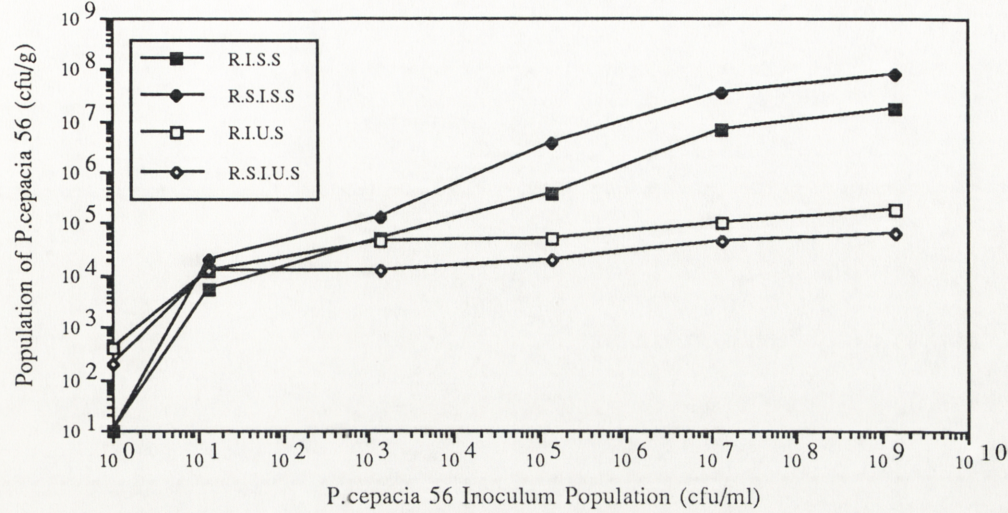
In the unsterilized soil, *Ps. cepacia* 56, *Ps. cepacia* 406 and *Ps. cepacia* 526 were also recovered from the rhizosphere soil and the root of *P. radiata* seedlings pre-inoculated with bacterial suspensions of various populations, although their populations were significantly smaller than those from equivalent treatments in sterilized soil. *Ps. cepacia* 56 and *Ps. cepacia* 406 showed the same patterns of recovery from the plant root and the rhizosphere soil as in the sterilized soil, i.e. their populations were became larger as the inoculum population increased. For *Ps. cepacia* 526, however, the population recovered from the plant root and the rhizosphere soil was significantly higher for initial inoculum level of $\times 10^9$ cfu/ml than for the other inoculum levels ($\times 10^1$ to $\times 10^7$ cfu/ml). There were no significant differences in the populations recovered between the inoculum levels of $\times 10^1$, $\times 10^3$, $\times 10^5$ and $\times 10^7$ cfu/ml.

Fig. 7.1. Recovery of *Ps. cepacia* strains from inoculated seeds



P. radiata

Fig. 7.2. Recovery of *Ps. cepacia* 56 from plant roots and rhizosphere soils.



- R.I.S.S ---- Root in sterilized soil
- R.S.I.S.S -- Rhizosphere soil in sterilized soil
- R.I.U.S ---- Root in unsterilized soil
- R.S.I.U.S -- Rhizosphere soil in unsterilized soil

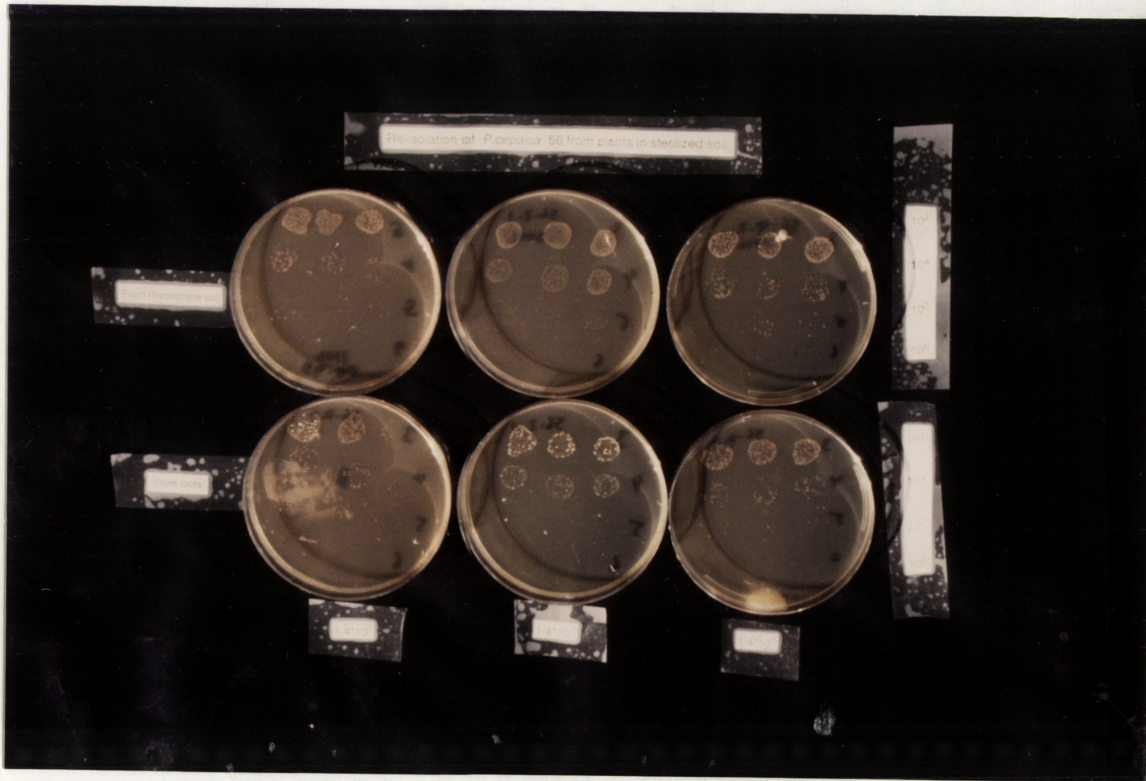
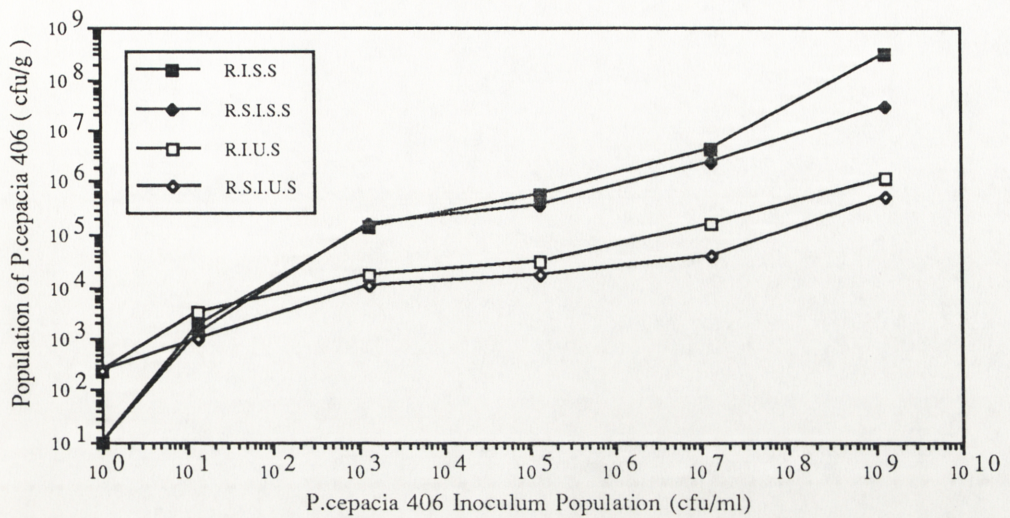


Fig. 7.3. Recovery of *Ps. cepacia* 406 from ~~plant roots~~ ^{*P. radiata*} and rhizosphere soils.

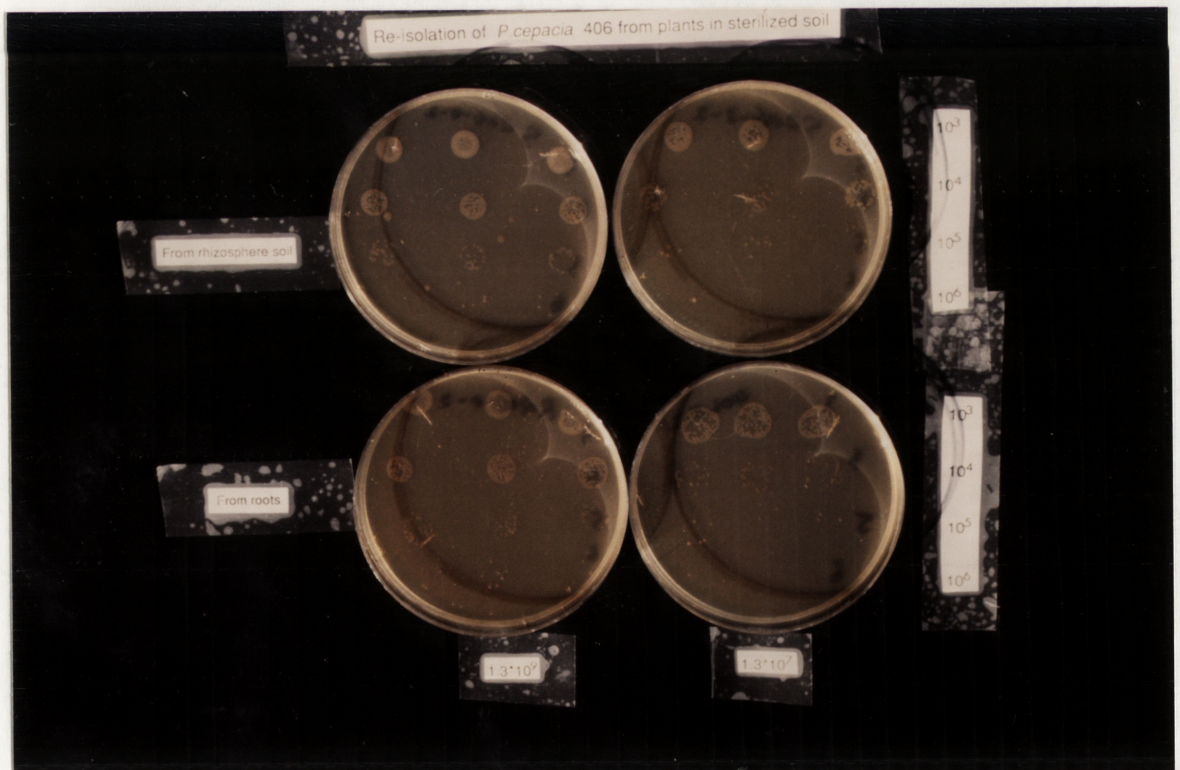


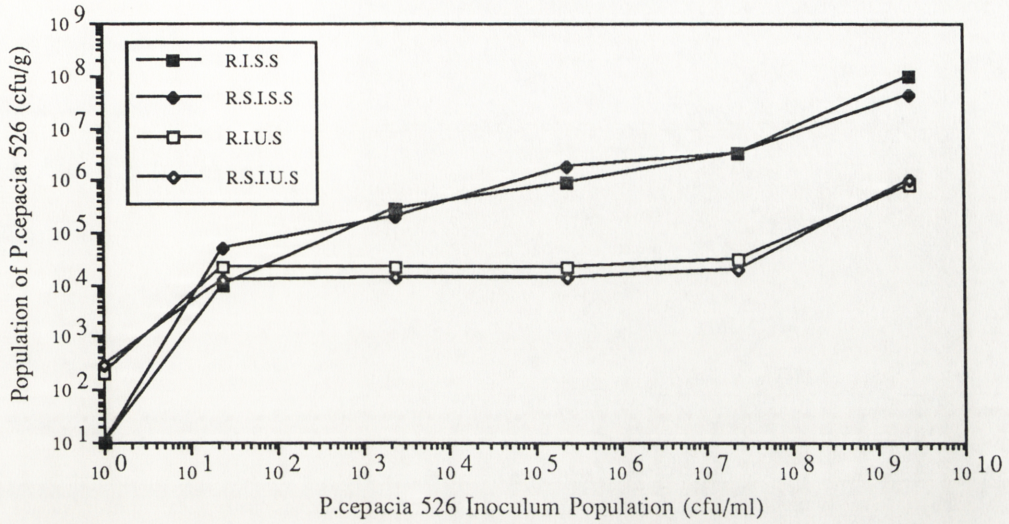
R.I.S.S ---- Root in sterilized soil

R.S.I.S.S -- Rhizosphere soil in sterilized soil

R.I.U.S ---- Root in unsterilized soil

R.S.I.U.S -- Rhizosphere soil in unsterilized soil



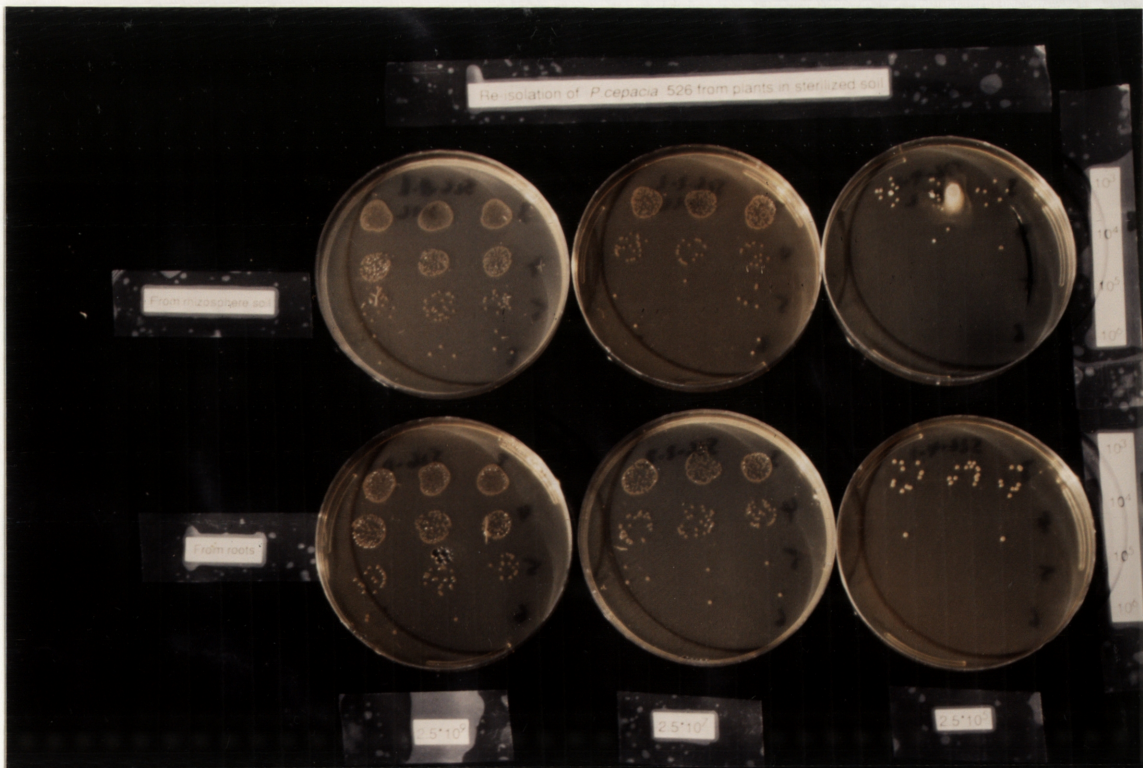
*P. radiata*Fig. 7.4. Recovery of *Ps. cepacia* 526 from plant roots and rhizosphere soils.

R.I.S.S ---- Root in sterilized soil

R.S.I.S.S -- Rhizosphere soil in sterilized soil

R.I.U.S ---- Root in unsterilized soil

R.S.I.U.S -- Rhizosphere soil in unsterilized soil



In the unsterilized soil, bacteria were also recovered from the control plants, with cultural characters similar to *Ps. cepacia*. However, the population was significantly smaller than the populations recovered from the *Ps. cepacia* inoculated plants.

7.3.2. Colonization and growth-promoting effects of *Ps. cepacia* 526 on turnip seedlings.

Ps. cepacia 526 established large populations on the roots of inoculated turnip seedlings. It was so dominant on the roots that its populations were not significantly different from the total root bacterial populations (LSD, $P=0.01$). Its population was as much as 4.6×10^5 cfu/g root within one week after sowing, and increased to the significantly higher levels of 7.1×10^6 cfu/g root in the second week and 1.8×10^7 cfu/g root in the third week and thereafter maintained at this level.

The total bacterial populations recovered from the uninoculated control seedling roots were significantly smaller than that from the inoculated seedling roots. Bacteria were also recovered on rifampicin nutrient agar plates from the uninoculated control seedling roots. These populations were maintaining at around 10^3 cfu/g root throughout the experiment, significantly smaller than those on the inoculated seedling roots (Fig. 7.5).

Ps. cepacia 526 significantly increased the plant root weight, from the first week through to the sixth week after sowing (Fig. 7.6). It was also noted that most *Ps. cepacia* 526 inoculated seedlings had more developed root system, including longer roots and more secondary and tertiary roots. The plant weight was also significantly increased after three weeks although not in the first two weeks (Fig. 7.7).

Insertion follows line 19: seedling roots (Fig. 7.5). These bacteria may represent those naturally exist in soil and are resistant to 100 mg/L rifampicin, for example, Pseudomonas fluorescens.

Fig. 7.5. Colonization of turnip seedling roots by *Ps. cepacia* 526.

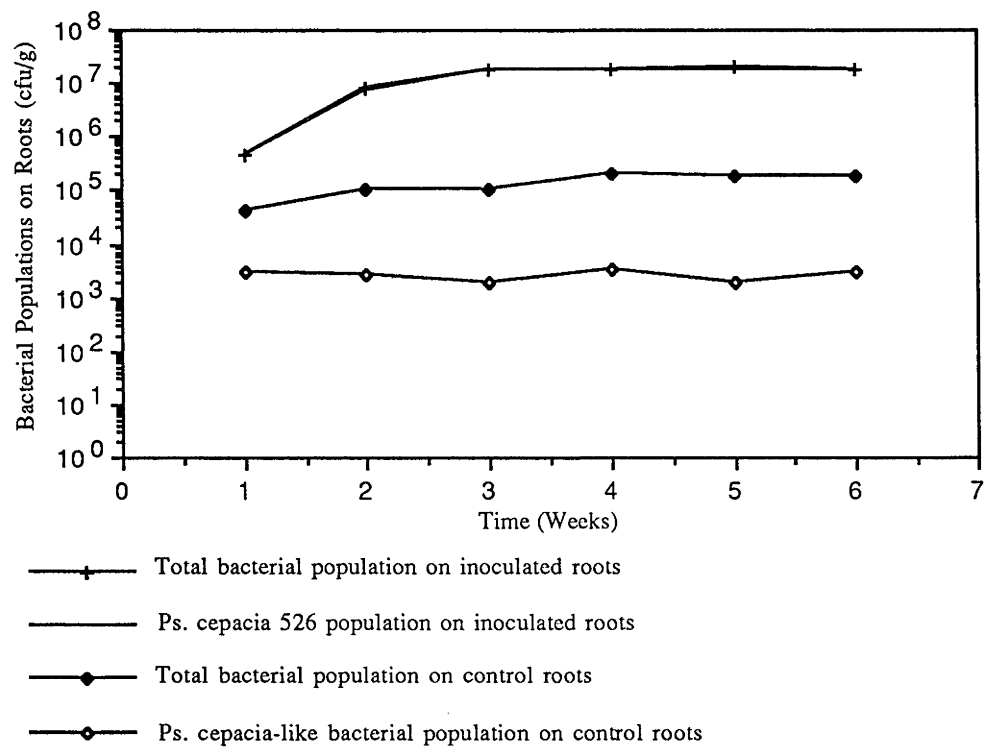


Fig. 7.6 Effect of *Ps. cepacia* 526 on turnip seedling root weight.

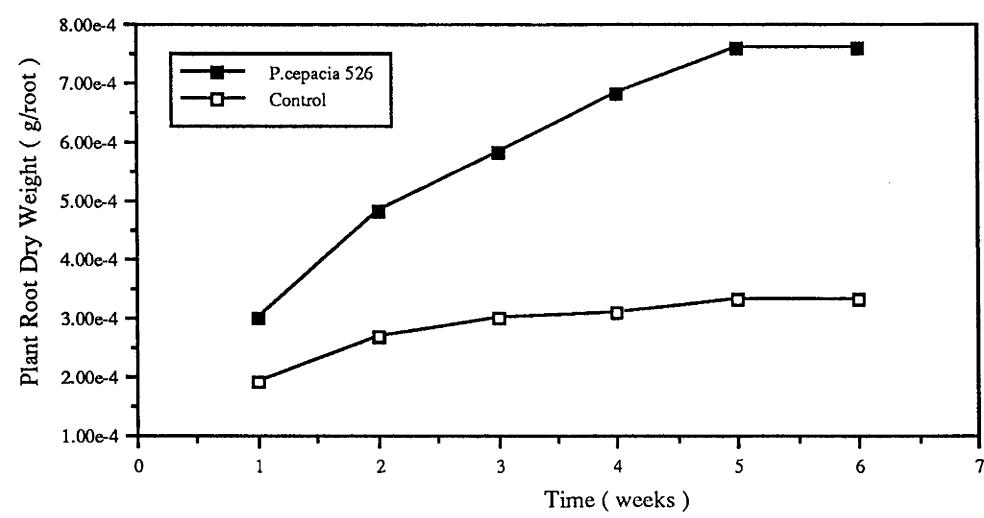
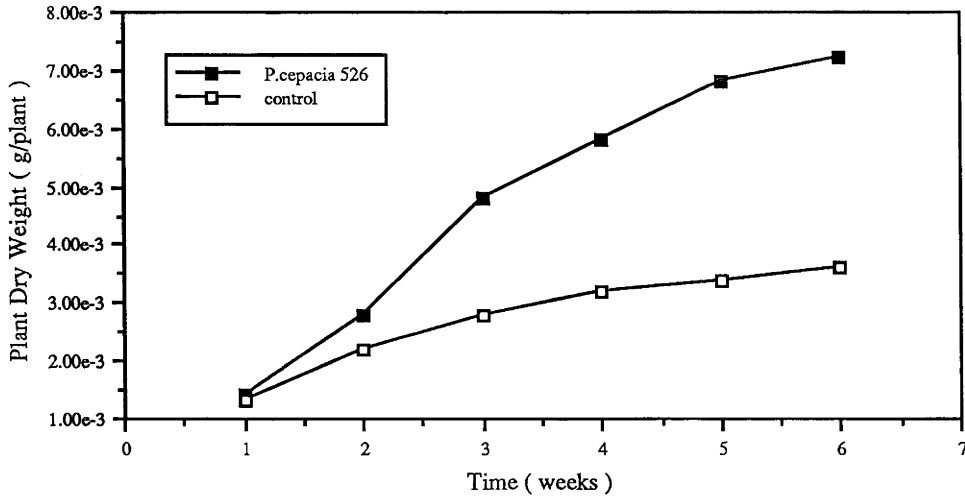


Fig. 7.7. Effect of *Ps. cepacia* 526 on turnip seedling weight.



7.4. Discussion and Conclusion.

The experiment with *P. radiata* aimed to study whether *Ps. cepacia* strains were able to establish high populations on *Pinus radiata* roots and in the rhizosphere soil and their associations with the initial inoculum populations. It was found that *Ps. cepacia* strains were able to colonize the plant root and the rhizosphere soil at low inoculum population ($\times 10^1$ cfu/ml) in the unsterilized soil as well as in the sterilized soil. It was also found that relative colonization was enhanced with increases in the inoculum population. Both the rhizosphere and the root populations of *Ps. cepacia* strains recovered from the sterilized soil were higher than that from unsterilized soil, indicating that the soil microorganisms in the unsterilized soil may have had strong effects on the *Ps. cepacia* strains.

The experiment confirmed the ability of *Ps. cepacia* to colonize the plant root and the rhizosphere soil and the effect of different inoculum populations. It was realized later, however, that more information could have been obtained and more conclusive conclusions made had the total soil microbial populations in the

non-rhizosphere soil, the rhizosphere soil and on the plant roots been examined. By comparing the *Ps. cepacia* population and the total soil microbial population, information concerning the competitive ability of *Ps. cepacia* ~~against the soil~~ *against other* microorganisms would have been provided.

In the experiment with turnip, the *Ps. cepacia* 526 population and the total bacterial population and their population dynamics over 6 weeks on the seedling root were examined. It was found that *Ps. cepacia* 526 was dominant on the inoculated turnip roots during the 6 week long experiment, with populations much larger than the total bacterial population in the control seedling roots. It was also found that *Ps. cepacia* 526 was able to promote turnip seedling growth by significantly increasing the seedling root weight and the seedling weight. The results indicated that the bacterium was able to compete effectively with the soil bacteria to colonize the turnip roots and to promote the plant growth. However, the results were based on the application of a high *Ps. cepacia* 526 inoculum population (1.5×10^9 cfu/ml) in the experiment. Such a high inoculum population ~~would~~ *would* ~~may~~ have put *Ps. cepacia* 526 at an unrealistically ~~advantaged competitive~~ *advantageous* position in relation to the soil microorganisms. Results obtained from experiments using lower *Ps. cepacia* 526 inoculum populations would be useful. An examination of the fungal populations on the plant root would also provide valuable information on the competition between *Ps. cepacia* 526 and the soil fungi.

To conclude, *Ps. cepacia* is capable of extensively colonizing both the rhizosphere soil and the plant root, and of promoting plant growth. Such capability is enhanced with increases in the inoculum population.

CHAPTER 8. GENERAL DISCUSSION AND CONCLUSION

The development of a plant disease management strategy and the successful application of a microorganism for plant disease control require a systematic study to obtain an understanding of the complex interactions between plant and plant pathogen, between plant and the potential biological control agent and between plant pathogen and the potential biological control agent. Correct understanding of the associations between these complex interactions and the soil environmental conditions are also critical.

The severity of turnip damping-off caused by *Ph. cinnamomi* is related to the pathogen population in soil but is greatly affected by soil temperature and matric potential. In the constant temperature room where the soil temperature was optimal for *Ph. cinnamomi*, the fungus caused severe disease at all soil matric potentials tested, with no significant difference existing between them, even when the *Ph. cinnamomi* inoculum level was as low as 0.2%. The disease became ~~severer~~ ^{more severe} as the inoculum levels increased (Chapter 3). On the other hand, in the glasshouse where the soil temperature was not favorable, *Ph. cinnamomi* used at 1% and 1.5% caused disease only in saturated soil (Chapter 3).

Ph. cinnamomi can infect plants by mycelium and zoospores (Marx & Bryan 1969, Malajczuk & Theodorou 1979). Both may be affected by soil temperature and soil matric potential. The fungus produced sporangia and zoospores only in saturated soil, even when soil temperatures were optimal (Chapter 6). Any damage caused by the fungus in non-saturated soils, including disease incidence, plant weight reduction and plant root weight reduction ^{would} ~~may~~ therefore originate from mycelial infection. Zoospores may cause such damage only in saturated soil. The possibility that initial infection at 0 kPa by *Ph. cinnamomi* may also be from mycelia cannot be excluded.

In the experiments reported in this thesis, *Ph. cinnamomi* mycelial activity was not directly influenced by soil matric potentials, ^{tested} but was greatly affected by soil temperature (Chapter 6). The plant root was of course the primary and most vulnerable infection site but damage there may be compensated for, wholly or partially, by further root development. Changes in plant weight and disease incidence do not therefore run in parallel with change in root weight. Plant root weight was therefore the most sensitive indication of damage caused by *Ph. cinnamomi*. The results of *Ph. cinnamomi* infection through mycelium at all the matric potentials tested may result in damage varying from none to root weight reduction and then to plant weight reduction and plant death, depending on the suitability of the soil temperature. When the soil temperature was suitable, such as in the constant temperature room, mycelial infection expressed its whole pathogenic potential so as to cause plant death at all matric potentials tested. When the soil temperature was less favorable, such as at 0, -1.5, -3.0 and -5.0 kPa (32.0°C, 32.5°C, 37°C, and 37°C, respectively) in the glasshouse, mycelial infection was slowed down but was not completely stopped, resulting in less damage to the plant such as root weight reduction. When soil temperature was unfavorable or even inhibitory, such as those at -10.0 kPa (38.5°C) and -15.0 kPa (39.5°C) in the glasshouse, the mycelia in soil might have been completely inactivated and therefore caused no infection or damage.

Turnip seedling survival and plant weight were significantly reduced in saturated soil, indicating a plant stress (Chapter 3). It is therefore very likely that such stress on the plant contributed to the disease incidence in saturated soil. Soil saturation may have directly increased the disease severity by predisposing the seedlings to infection caused by both mycelia and zoospores of *Ph. cinnamomi*. Such predisposing effects may be particularly important when

Ph. cinnamomi mycelial activity or the production of zoospores are adversely affected by unfavorable soil temperature.

Complex interactions exist between air temperature, soil matric potential and soil temperature (Chapter 3). The temperature regime of a particular soil may be very different at various soil water contents (which is associated with different matric potentials) in one location with a particular range of air temperatures, or at the same water content but at different locations with different air temperatures. For example, the temperatures of soils at various matric potentials in the glasshouse differed from one another because of their different water contents, and the temperature of soil in the glasshouse and that in the constant temperature room at the same matric potentials were also different.

Such complex interactions between air temperature, soil matric potential and soil temperature are important in determining the disease incidence caused by *Ph. cinnamomi* and may account for the conflicting results and conclusions commonly found in the previous literatures (refer to 1.2). It is therefore important that, in the design of experiments and in the interpretation of results, attention must also be given to these interactions rather than a particular variable.

Had the experiments been performed at only one temperature range (that unfavorable in the glasshouse or that favorable in the constant temperature room), this study would have come to conclusion which may have been partially true or may be very different. For instance, if the experiments had been conducted only in the glasshouse, the conclusion would have been made that the soil matric potential is the most important factor in deciding the disease severity and that *Ph. cinnamomi* disease only occurred in saturated soil due to enhanced sporulation. Even if the mycelial growth had been examined, the conclusion

could have been that the low matric potential, but not the soil temperature, restricted the mycelial growth and infection. The conclusions would have exaggerated the importance of soil saturation and increased sporulation. It is therefore not surprising that most researchers found that disease caused by *Ph. cinnamomi* were ~~severer~~ ^{more severe} in wet soil than in dry soil and held the enhanced sporulation in wet soil to be responsible (refer 1.2). It is probably why Weste and her colleagues concluded that during the hot summer, the low soil matric potentials restricted mycelial growth and sporangial production by *Ph. cinnamomi* (Weste & Taylor 1971, Weste & Vithanage 1977). In fact, the high soil temperature may have been the critical variable. It is therefore important that experiments be carried out at various soil temperature ranges as well as with different soil moisture regimes so that an overview of the interactions between soil temperature and soil moisture can be obtained.

Mycelial growth of *Py. ultimum* and the severity of turnip damping-off caused by the fungus is not affected by the range of soil temperatures and soil matric potentials used (Chapters 3 & 6). Increases in the fungal inoculum population also increased damage (Chapter 3). This suggests that, within the range of soil temperatures and soil matric potentials used, the pathogenicity of *Py. ultimum* is not affected in the presence of a susceptible host plant. Severe disease incidence is probably due to the high susceptibility of the plant or the great virulence of the fungus or both. The importance of host susceptibility in disease caused by *Py. ultimum* was supported by many previous reports indicating that soil matric potentials did not affect disease severity through its direct effects on the pathogen, but through indirect effects on the host plant involving changes in the amount of root exudates (Kerr 1964, Osburn & Schroth 1986, Schroth and Cook 1964, Stanghellini et al. 1971).

To obtain the antagonists of plant pathogens through large-scale screening of a large number of microorganisms from soil is an important step in the development of plant disease biological control, when there is no known potential microorganism available. It is not possible to test antagonism between candidate isolates and plant pathogen in soil due to the workload involved and the lack of reliable methods. Instead, culture tests have been used as a shortcut, being quicker and easier. However, because the culture test conditions do not accurately represent the full range of variables, nor of their interactions, the results are not always reliable predictors of field results. It is therefore important that culture tests be performed under various conditions and using different methods, especially when a microorganism tested may produce a number of antibiotics against a range of pathogens. The expression of the inhibitions will depend on the method used (refer to chapter 4, section 4.4). The results obtained from culture tests should also be confirmed by experiments in natural soil. Study of the interactions between *Ph. cinnamomi* and *Ps. cepacia* 526, between *Py. ultimum* and *Ps. cepacia* 526 in this thesis were based on these principles.

Antibiotic inhibitions can occur mutually between a plant pathogen and a biological control agent. While the biological control agent may inhibit the growth, reproduction and survival of a pathogen, the plant pathogen may also inhibit the biological control agent. Action by the plant pathogen may result in the ineffectiveness of the potential biological control agent in controlling the disease.

Ph. cinnamomi inhibited *Ps. cepacia* 526 in both agar and liquid media (Chapter 5). ^{Indirect}~~Circumstantial~~ evidences strongly suggested that the inhibition was related to the production of short-lived antibiotics by the fungus at least on agar media. It is possible that *Ph. cinnamomi* may also have produced short-lived

antibiotics in liquid medium as it did on agar media (Chapter 5). Contents of the media nutrient were critical for the occurrence of the antibiotic effect of *Ph. cinnamomi* on *Ps. cepacia* 526 and thiamine was the essential component (Chapters 4 & 5). Although this nutrient effect has not been studied in soil, it is anticipated that the oat seeds on which *Ph. cinnamomi* inoculum was produced contained thiamine (Chapter 3) and may have played an important role in the inhibition of *Ps. cepacia* 526 by the fungus.

Py. ultimum did not inhibit the growth of *Ps. cepacia* 526 on any media tested, but was itself inhibited by the bacterium in both agar and liquid media. Also, *Ps. cepacia* 526 tended to move toward ~~the~~ *Py. ultimum* and quickly multiplied ~~itself~~, perhaps indicating a stimulatory effect by the fungus.

Ph. cinnamomi and *Py. ultimum* therefore vary considerably in their effects on *Ps. cepacia* 526. The postulated antibiotic produced by *Ph. cinnamomi* seems important in the interaction between the fungus and *Ps. cepacia* 526. Inhibition by *Ph. cinnamomi* probably resulted in the failure~~s~~ of the bacterium to move and multiply along the fungal hyphae in culture (Chapter 5), to affect the fungal mycelial growth and sporulation in both culture and soil (Chapter 6), and consequently to control the damping-off caused by the fungus (Chapter 3). In contrast, *Py. ultimum* did not produce any antibiotics but probably produced stimulatory substance. This resulted in quick movement and multiplication of the bacterium along the fungal hyphae in culture (Chapter 5), increased~~ed of the~~ fungal hyphal lysis in soil (Chapter 6), and in the effectiveness of the bacterium to control the damping-off caused by the fungus (Chapter 3).

Ps. cepacia 526 has been found to produced three antibiotics (pyrrolnitrin, aminopyrrolnitrin and xylocandin, Hebbar 1986, Dr P.J. Dart & Mr R. Rickards, personal communications). Pyrrolnitrin ~~inhibit~~ ^{inhibited} neither *Ph. cinnamomi* nor *Py. ultimum* (Chapter 5). This suggests that pyrrolnitrin could not have been

responsible for the antibiotic interactions between the bacterium and the fungi in culture and probably also in soil. Neither aminopyrrolnitrin nor xylocandin was tested because of unavailability of the antibiotics. There is hence a possibility that *Ph. cinnamomi* and *Py. ultimum* may have been affected differently by these antibiotics produced by *Ps. cepacia* 526, resulting in differences in inhibition of fungal growth and sporulation and disease control. An antibiotic produced in culture may not be produced in soil, and may not have the same inhibition against a pathogen in soil as it has in culture. It is possible that *Ps. cepacia* 526 ^{the} antibiotic inhibitory to *Ph. cinnamomi* in culture may not have been produced or, if produced, may have lost its effect in soil, resulting in failure in disease control. Howell & Stipanovic (1979, 1980) found that pyrrolnitrin and pyoluteorin were responsible for the different inhibitions by *Pseudomonas fluorescens* against *Rhizoctonia solani* and *Pythium ultimum*. Pyrrolnitrin was strongly inhibitory to *Rh. solani*, but not to *Py. ultimum* (Howell & Stipanovic 1979); whereas pyoluteorin was found to be inhibitory to *Py. ultimum*, but not to *Rh. solani* (Howell & Stipanovic 1980). Pyrrolnitrin is not readily diffusible and is released upon bacterial cell lysis, but is persistent in the soil (Howell & Stipanovic 1979), whereas pyoluteorin was ~~absorbed~~ ^{adsorbed} and inactivated by the soil colloids when added directly to soil (Howell & Stipanovic 1980).

The ability of a potential biological agent to promote plant growth can contribute to reduced disease severity. Such ability is especially important in a pathosystem where the pathogen is not very virulent and the disease incidence is significantly determined by plant resistance subject to environmental stress. While unfavorable environmental conditions may predispose the host plant to infection by the pathogen, the microorganism may increase the plant resistance or simply offset the damage caused by the pathogen by increasing plant growth, although it may not necessarily act directly against the pathogen. In a

pathosystem where the pathogen is very virulent and not affected by soil condition, however, the limited growth-promoting effects of the microorganism on the host may not be enough. *Ps. cepacia* 526 has good capability to improve turnip plant growth resulting in increased plant root weight and plant weight (Chapters 3 & 6). Its ability to increase turnip seed germination and plant growth ~~result~~ ^{resulted} in increased seedling survival in saturated soil where the plant is stressed (Chapter 3), even in the presence of *Ph. cinnamomi*. In the presence of *Py. ultimum*, the growth promoting effect of *Ps. cepacia* 526 were less important or negligible.

For a microorganism to be capable of ~~inducing~~ ^{influencing} plant growth, it should also be established in the rhizosphere and on the plant root and form a significant part of the total microflora. Colonization is determined by the complex interactions between the microorganism and other soil microorganisms. The microorganism should efficiently compete ~~for~~ ^{for} and utilize the substrates exuded by the plant root or that existing in the soil. *Ps. cepacia* 526 apparently has the ability and is able to establish itself in the rhizosphere and on *P. radiata* roots, even when the inoculum used is as small as 10^1 cfu/ml. *Ps. cepacia* 526 is also able to compete effectively with ~~the~~ ^{other} soil bacteria to form the major part of the total microbial population on turnip roots (Chapter 6). Although *Ps. cepacia* 526 was not tested on turnip at low inoculum population, the results indicated that the application of a high *Ps. cepacia* 526 inoculum population might well be useful in increasing turnip plant growth. The combined results of chapters 3 and 6 suggested that *Ps. cepacia* 526 is able to compete with soil microorganisms, to colonize the rhizosphere and plant root and to promote plant growth.

The plant growth-promoting and the biological control effects of *Ps. cepacia* 526 were not affected by the soil temperatures and soil matric potentials

tested (Chapter 3), indicating good adaptability of the bacterium within these ranges.

Suggestions for future work

1. In experiments reported in this thesis, the influence of soil temperature and matric potential on various activities of *Ph. cinnamomi*, *Py. ultimum* and *Ps. cepacia* 526 have been investigated within relatively small ranges of these environmental variables. As already discussed in chapter 2 (refer to 2.4.3), it was expected that the matric potential differences within the range used would have had significant effects on *Ps. cepacia* 526 and, on the mycelial growth, sporangial production and zoospore release of the fungi. Soil temperatures and matric potentials used showed significantly differential effects on the mycelial growth and sporangial production and zoospore release of *Ph. cinnamomi*, and on the damping-off it caused. No such effects, however, were shown on *Py. ultimum*, nor on *Ps. cepacia* 526 in relation to its plant growth-promoting or disease control effects. A wider range of soil matric potentials may well have significant effects on these two microorganisms and therefore should be a subject for further studies. The circumstantial evidence, that high temperature affects infection by zoospores and mycelia of *Ph. cinnamomi* differently, (Chapters 3 & 6) requires confirmation by further experiments on the responses of these structures to high temperature.

2. Circumstantial evidence has shown that *Ph. cinnamomi* produced short-lived antibiotics, which inhibited *Ps. cepacia* 526 in vitro and may also have been responsible for the failure by the bacterium to control the disease effectively. The existence of these antibiotics needs to be confirmed and their properties characterized. Influence of nutrients especially thiamine on the

interaction between *Ph. cinnamomi* and *Ps. cepacia* 526 needs examination quantitatively in soil.

3. The effects of *Ps. cepacia* 526 on *Ph. cinnamomi* mycelial growth, sporangial production and zoospore release and on *Py. ultimum* mycelial growth have been studied. Further work should examine effects of the bacterium on the saprophytic growth and survival by the fungi in soil (chlamydospores and oospores).

4. The different effects of the antibiotics produced by *Ps. cepacia* 526 on *Py. ultimum* and *Ph. cinnamomi* need to be studied further in culture and in soil, when the antibiotics become available. The indication that *Py. ultimum* may have produced substances stimulatory to *Ps. cepacia* 526 requires further investigation. ^{a substance or}

5. The mechanisms by which *Ps. cepacia* 526 stimulated plant growth also require further studies.

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